Novel Treatments in Allergen-Specific Immunotherapy

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Julia M. Martínez Gómez

aus

Spanien

Promotionskomitee

Prof. Dr. Adriano Aguzzi (Vorsitz)

PD Dr. med. Thomas Kündig (Leitung der Dissertation)

Prof. Dr. Bruno Gander

Zürich, 2008

PREFACE

This thesis envisioned the development of novel strategies for improving the effectiveness of allergen-specific immunotherapy (SIT). The increased prevalence of allergic disorders among the population in industrialized countries has encouraged scientists to investigate the basic mechanisms of allergy and has led to a re-evaluation of existing treatments.

Almost hundred years have past since Freeman and Noon first described SIT. They subcutaneously injected hay fever allergic patients with increasing amounts of grass pollen extract. These incremental regime, which was necessary to minimize the risk of allergic reactions, still constitutes the basic principle of the current treatment, typically involving numerous injections over years.

Although SIT is the only curative treatment with long-lasting effect and the potential to stop progression of the disease, less than 5 % of allergic patients undergo SIT. Over the last decades, several means to improve SIT safety and efficacy and, therefore, patient compliance have been investigated. My work focussed on three different approaches to improve the existing therapy: (i) the route of allergen administration, (ii) the use of modified recombinant allergen molecules, and (iii) the use of a polymer-based allergen delivery system with adjuvant potential.

The use of a more efficient route of antigen administration such as the intranodal route could help to reduce the number of injections required for SIT. Moreover, intranodal injections require lower allergen doses to induce strong immune responses, reducing the risk of allergic reactions. The injection in subcutaneous lymph nodes is technically feasible and has already been performed in several clinical trials. The only additional requirement to standard SIT is ultrasound guidance.

Modified recombinant allergens targeted to the MHC-II antigen presentation pathway via a truncated invariant chain sequence fused to the allergen, increases their immunogenicity and reduces human basophil degranulation as compared to the wild type recombinant allergen. Such allergens increase the efficacy without increasing the risk of allergic reactions.

The combination of more immunogenic allergens with the more efficient intranodal route represents a promising tool to improve SIT and is planned to be tested soon in a phase II clinical trial.

Particulate delivery systems such as those made of poly(lactide-co-glycolide) (PLGA) show great potential for SIT, although their application to the clinics would still require further research. The capacity of PLGA to deliver antigens over prolonged periods of time could mimic the repeated booster injections of SIT. In addition to the depot effect, smaller PLGA particles (smaller than 10 μm) can be taken up by antigen presenting cells and thereby deliver allergens directly to lymphoid tissues. Furthermore, the co-encapsulation of other adjuvants can enhance and modulate immune responses.

This thesis shows that the administration of allergens directly into secondary lymphoid organs, the use of modified recombinant allergens as well as the use of polymeric biodegradable microparticles for delivery of allergens, are all promising approaches to improve the current SIT treatment.

TABLE OF CONTENTS

SUMMARY

Nowadays, type I hypersensitivity reactions affect 20-30 % of the population in developed countries. Such reactions are associated with T helper 2 (Th2) cells and are mediated by IgE antibodies against substances, so called allergens, otherwise innocuous to the immune system. Pharmacotherapy is the most used treatment among allergic patients but it only ameliorates the symptoms without treating the cause of the disease. The only curative treatment for allergy patients that has a long lasting effect and that can stop progression of disease is allergen-specific immunotherapy (SIT). However, only 3-4 % of patients undergo SIT mainly because of three reasons: (1) the long duration of the treatment, which usually involves 30-80 subcutaneous injections over a time period of 3-5 years, (2) the risk of allergic reactions, and (3) its high associated cost. Improving SIT by lowering the risk of adverse reactions and the number of injections would represent a significant advantage in terms of patient compliance and would also have socio-economical implications.

Over the last decades efforts to improve SIT have focussed on optimizing the allergen molecules, their galenics including particulate delivery systems, such as virus like particles or poly(lactide-co-glycolide), the adjuvants, and more convenient routes of administration, i.e. oral and sublingual allergen administration.

My PhD aimed at developing novel therapeutic strategies for SIT. In the first approach we tried to improve SIT by direct intralymphatic allergen administration (**Chapter 2**), in the second approach we used modified recombinant allergens (**Chapter 3**) and in the third approach we used polymeric biodegradable microparticles as an allergen delivery system (**Chapter 4**).

In the first chapter of this thesis we investigated an alternative route for allergen administration. The geographical concept of immune reactivity postulates that for an immune response to occur antigens need to reach secondary lymphoid organs. Therefore, we proposed the direct administration of allergens into lymph nodes to stimulate the immune system more efficiently. In fact, we showed that intralymphatic injections induce stronger immune responses than the current used subcutaneous injections even with lower allergen doses. Additionally, only intranodal injections induced the Th1-associated antibody subclass IgG2a. Hence, by using the intranodal route of administration SIT can be shortened and the allergen dose lowered, which reduces the risk of allergic side effects.

The second project of this thesis tested the potential of targeting the MHC-II presentation pathway for allergy vaccines. The recently developed modular antigen transporter (MAT) allergens have a sequence derived from the Tat protein of HIV-1 for intracellular translocation and the first 110 amino acids from the human invariant chain targeting allergens to the MHC class II presentation pathway. We used the major cat allergen Fel d 1 as a model allergen and found increased immunogenicity with long-lasting Th1 associated antibodies and cytokines after three intralymphatic injections in mice immunized with MAT-Fel d 1 allergen compared to the normal recombinant allergen or the cat fur extract. Correspondingly, in a therapeutic model, mice desensitized with the modified allergens were better protected against anaphylaxis after a challenge with a high dose of the cat fur extract. MAT allergens increased the presentation efficiency without the need of increasing the allergen dose which would provoke adverse allergic reactions. Moreover, these modified allergens reduced basophil degranulation of cat allergic patients. This hypoallergenicity of the MAT-Fel d 1 allergen seemed to be explained by both its reduced IgE binding capacity and by the rapid internalization of the molecule due to the Tat sequence.

In the third project, we studied the adjuvant capacity of poly (lactide-co-glycolide) (PLGA) microparticles. We encapsulated the major bee venom allergen, phospholipase A2 (PLA2) as a model allergen, together with an immunomodulator, i.e. unmethylated CpG oligodeoxinucleotide, which is a known Th1 response inducer. PLGA are biocompatible and biodegradable polymers that are approved for use in humans. Moreover, they are attractive candidates for allergy vaccines because they release the encapsulated proteins over prolonged periods of time and would thereby reduce the need of numerous booster injections. In addition, entrapped allergen is less accessible for binding IgE on the surface of mast cells and basophils, reducing the risk of allergic reactions and anaphylaxis. In this study, we found that microparticles in combination with CpG were strongly immunogenic after two injections and were able to shift immune responses towards a Th1 type. The encapsulation of CpG proved to enhance immunogenicity as compared to admixed CpG. Additionally, co-encapsulation of protamine, used to stabilize CpG, further increased immunogenicity. The strong Th1 responses correlated with enhanced protection against anaphylaxis in bee venom allergic mice. These results indicate the potential of such formulations for use in SIT.

Over the past years, PLGA formulations with surface modifications have been described for delivery of antigens or adjuvants. We prepared an additional PLGA

formulation with encapsulated PLA2 and with protamine as a coating agent for adsorption of CpG to the microparticle surface. The immunogenicity of such coated particles with admixed CpG was similar to co-encapsulated CpG and protamine. However, in the absence of CpG, this formulation, unlike the uncoated particles, was able to induce a strong immune response. These results prompted us to study the effects of protamine-coated microparticles in different experimental settings and became an extra project of this thesis (**Chapter 5**). Interestingly, we always found increased T- and B-cell responses *in vivo* when protamine-coated particles were used as well as enhanced particle uptake and antigen presentation *in vitro*. Protamines are small arginine-rich proteins that share great similarity with sequences from certain viral proteins such as Tat from HIV-1 and VP22 from herpes simplex virus. These sequences have a known translocation activity, which was also reported for protamine in 2005 by two independent groups. We therefore hypothesized that protamine could have a similar translocation activity on our coated microparticles. In fact, protamine-coated microparticles containing plasmid coding for green-fluorescence protein were able to transfect the non-phagocytic HEK cell line. Additional research is needed to characterize such protamine-coated particles in order to develop further a new technology combining the advantages of microparticles for prolonged antigen release and protamine for facilitating cell penetration of the delivery system. The potential applications of such intracellular delivery system technology may span the areas of vaccine development, cancer immunotherapy, and gene/protein delivery.

In conclusion, the novel SIT approaches presented in this work could represent an alternative to the current SIT treatment with reduced number of injections and lower risk of adverse events. In particular, the project with MAT allergens served to set up the basis for further clinical research in which a phase II clinical trial is planned in the near future.

ZUSAMMENFASSUNG

Heute sind rund 20-30% der Bevölkerung industrialisierter Länder von Sofort-Typ Allergien betroffen. Diese Form der Hypersensitivitätsreaktionen entsteht dann, wenn das Immunsystem gegen ansonsten harmlose Substanzen mit der Bildung von T2-Helferzellen (Th2) reagiert, wodurch es zur Bildung von Antikörpern der Klasse IgE kommt.

Die einzige kausale Therapie, die einen lang anhaltenden Effekt erzielen und das Fortschreiten der Krankheit aufhalten kann, ist die Allergen-spezifische-Immuntherapie (SIT). Dennoch unterziehen sich nur 3-4 % der Patienten dieser Therapie, was hauptsächlich auf die folgenden 3 Gründe zurückzuführen ist: (1) die lange Dauer der Behandlung, sie benötigt normalerweise 30-80 subkutane Injektionen über einen Zeitraum von 3-5 Jahren, (2) das Risiko allergischer Reaktionen und (3) die mit der Behandlung verbundenen hohen Kosten. Eine Verbesserung der SIT durch Verminderung der Nebenwirkungen und der Anzahl nötiger Injektionen würde zu einem enormen Vorteil bezüglich der Patienten Compliance führen und hätte auch sozioökonomische Vorteile.

Um die SIT zu verbessern wurde in den letzten Jahrzehnten der Fokus vor allem auf eine Optimierung von Allergenmolekülen und ihrer Galenik mitsamt partikelartiger Trägersysteme gelegt, wie beispielsweise virus-ähnliche oder Poly(lactide-co-glycolide) Partikel, sowie auf eine Optimierung von Adjuvantien und geeigneteren Verabreichungsmethoden, z.B. sublingual oder oral.

Meine Doktorarbeit hatte das Ziel neue therapeutische Strategien für die SIT zu entwickeln. In einem ersten Ansatz versuchten wir die SIT durch intralymphatische Verabreichung der Allergene zu verbessern (Kapitel 2), in einem zweiten Ansatz verwendeten wir modifizierte rekombinante Allergene (Kapitel 3) und in einem dritten Ansatz verwendeten wir polymere, biologisch abbaubare Mikropartikel als Allergenträgersystem (Kapitel 4).

Im ersten Kapitel dieser Doktorarbeit untersuchten wir alternative Wege der Allergenverabreichung. Antigene müssen die sekundären Lymphorgane erreichen, damit eine Immunreaktion ausgelöst wird. Deswegen versuchten wir durch direkte Verabreichung der Allergene in die Lymphknoten das Immunsystem effektiver zu stimulieren. Wir konnten tatsächlich zeigen, dass intralymphatische Injektionen stärkere Immunantworten auslösen als die klassischerweise verwendeten subkutanen Injektionen, selbst bei niedrigerer Dosierung der Allergene.

Ausserdem löste nur die Injektion in die Lymphknoten die Produktion von IgG2a Antikörpern aus, welche Th1-abhängig sind. Demzufolge kann die Dauer der SIT durch direkte Injektion der Allergene in die Lymphknoten verkürzt und gleichzeitig die Dosis der Allergene erniedrigt werden, wodurch das Risiko von Nebenwirkungen verringert wird.

Im zweiten Projekt dieser Doktorarbeit versuchten wir die zur SIT verwendeten Allergenmoleküle so zu modifizieren, dass sie verstärkt auf MHC Molekülen der Klasse II präsentiert werden. Die vor kurzem entwickelten „modularen Antigentransporter“ (MAT) Allergene tragen eine Sequenz, die sich vom Tat Protein des HIV-1 ableitet, um so die intrazelluläre Translokation zu steuern. Weiterhin enthalten sie die ersten 110 Aminosäuren der menschlichen invarianten Kette, die das Allergen in den MHC-II Signalweg leitet.

Als Allergiemodell verwendeten wir das Hauptallergen aus Katzenhaar, Fel d 1. Verglichen mit dem normalen rekombinanten Allergen oder Katzenhaarextrakt zeigte die Injektion von MAT- Fel d 1 eine erhöhte Immunogenität in Mäusen.

Dementsprechend konnten wir auch Mäuse, welche zuvor mit Katzenhaarextrakt sensibilisiert wurden, mit MAT-Fel d 1 effizienter desensibilisieren als mit nicht modifiziertem Fel d 1 oder mit Katzenhaarextrakt. Nur die mit MAT-Fel d 1 desensibilisierten Mäuse waren bei einer Provokation mit dem Katzenhaarextrakt gegen einen anaphylaktischen Schock geschützt. MAT Allergene erhöhten die Präsentationseffizienz ohne eine erhöhte Allergendosis zu benötigen, welche allergische Nebenwirkungen hervorrufen würde. Zudem reduzierten diese modifizierten Allergene die Degranulation von basophilen Granulozyten aus Patienten mit Katzenallergie. Diese hypoallergene Wirkung des MAT-Fel d 1 Allergenmoleküls schien durch seine reduzierte IgE Bindungskapazität und die rasche Internalisierung des Moleküls aufgrund seiner Tat Sequenz verursacht zu werden.

Das dritte Projekt untersuchte die adjuvanten Eigenschaften von Poly(lactide-co-glycolide) (PLGA) Mikropartikeln. Als Modellallergen vekapselten wir das Hauptallergen des Bienengifts, Phospholipase A2 (PLA2), zusammen mit nicht methylierten CpG-Oligodeoxinukleotiden. Solche CpG-Nukleotide sind Immunmodulatoren, die für die Induktion von Th1-Reaktionen bekannt sind. PLGA sind biologisch kompatible und abbaubare Polymere, die für eine Verwendung im Menschen zugelassen sind. Zudem erscheint die Verwendung von PLGA für die SIT interessant, da PLGA die verkapselten Allergene über einen längeren Zeitraum hinweg freisetzen, wodurch möglicherweise die Anzahl der benötigten Allergeninjektionen reduziert werden könnte. Weiterhin sind verkapselte Allergene schlechter zugänglich für die Bindung an IgE, das auf der Oberfläche

von Mastzellen und basophilen Granulozyten gebunden ist, wodurch das Risiko von allergischen Reaktionen, inklusive der Anaphylaxie, reduziert wird. In dieser Studie zeigten wir, dass mit CpG kombinierte Mikropartikel nach zwei Injektionen bereits stark immunogen wirkten und die Immunreaktion in Richtung Th1 verschoben. Verglichen mit zugemischtem CpG erhöhte die Verkapslung von CpG die Immunogenität. Durch die zusätzliche Verkapslung von Protamin, das zur Stabilisierung von CpG dient, wurde die Immunogenität noch weiter verstärkt. In Mäusen mit Bienengiftallergie wurde eine Korrelation zwischen der starken Th1-Antwort und einem erhöhtem Schutz vor einer Anaphylaxie nachgewiesen. Diese Ergebnisse weisen auf das Potential solcher Formulierungen für den Einsatz in SIT hin.

In den letzten Jahren wurden PLGA Formulierungen mit Oberflächenmodifikationen für den Transport von Antigenen oder Adjuvantien beschrieben. Wir stellten eine weitere PLGA Formulierung mit verkapseltem PLA2 her, die Protamin als beschichtende Substanz für die Adsorption von CpG an die Mikropartikeloberfläche enthielt. Die Immunogenität dieser beschichteten Partikel mit zugesetztem CpG war vergleichbar mit der von co-verkapseltem CpG und Protamin. Im Gegensatz zu nicht beschichteten Partikeln konnte diese Formulierung jedoch auch in Abwesenheit von CpG eine starke Immunantwort induzieren. Diese Ergebnisse regten uns dazu an, die Effekte von protamin-beschichteten Mikropartikeln in verschiedenen experimentellen Fragestellungen zu untersuchen. Dadurch entstand ein zusätzliches Projekt in dieser Arbeit (Kapitel 5). Interessanterweise detektierten wir bei der Verwendung von protamin-beschichteten Partikeln immer erhöhte T- und B-Zellantworten *in vivo* und eine erhöhte Partikelaufnahme und Antigenpräsentation *in vitro*. Protamine sind kleine argininreiche Proteine, die eine grosse Ähnlichkeit mit Sequenzen bestimmter viraler Proteine wie Tat von HIV-1 und VP22 von Herpes Simplex Virus aufweisen. Diese Sequenzen besitzen eine bekannte Translokationsaktivität, die 2005 von zwei unabhängigen Gruppen auch für Protamin berichtet wurde. Wir stellten daher die Hypothese auf, dass Protamin auf unsere beschichteten Mikropartikel eine ähnliche Translokationsaktivität ausübt. Tatsächlich konnte eine nicht phagozytierende HEK Zelllinie mit protamin-beschichteten Mikropartikeln, die ein für das grün fluoreszierende Protein codierendes Plasmid enthielten, transfiziert werden. Die Anwendung einer solchen Technologie zum intrazellulären Transport wäre in der Entwicklung von Vakzinen, in der Krebsimmuntherapie und bei Gen- bzw. Protein-Transporten denkbar.

Schlussendlich könnten die in dieser Arbeit vorgestellten neuen SIT Ansätze, die mit einer reduzierten Anzahl Injektionen und einem geringeren Risiko von Nebenwirkungen verbunden sind, eine Alternative zur aktuellen SIT Behandlung darstellen. Das Projekt mit MAT Allergenen diente bereits dazu, eine Grundlage für weitere klinische Forschungen zu schaffen. Zur Zeit findet eine klinische Studie der Phase I/IIa statt.

CHAPTER 1

Immunotherapeutic targeting of allergy

Part I published in Inflammation & Allergy – Drug Targets, (2006) 5 : 243-352

INTRODUCTION

The term allergy was introduced more than a hundred years ago, and usually refers to immediate type hypersensitivity reactions according to the classification of Coombes and Gell in 1963, which today are known to be IgE mediated. Over the last 20 years, the prevalence of allergic rhinitis and rhino-conjunctivitis in industrialized countries has doubled (Durham et al., 1999b; Schoenwetter et al., 2004). The cumulative prevalence rate varies both within and between different countries, with studies indicating a 20% rate in the US and an average 23% prevalence in a population-based survey in Belgium, France, Germany, Italy, Spain and the UK (Bauchau and Durham, 2004).

Several genetic and non-genetic factors have been identified to play a role in the development of allergies. Among the genes described to be involved in allergy susceptibility are those on chromosome 2 encoding for CD28, and CTLA-4, as well as those on chromosome 5 encoding for IL-3, IL-4, IL-5, IL-9, IL-13, GM-CSF and leukotriene C_4 synthase (Steinke et al., 2008). On the other hand, environmental factors such as endotoxin exposure, air pollution and tobacco smoke have also been described to influence the onset of allergic diseases (Thomson, 2007). Likewise, the route of exposure, the dose of the allergen and some of its structural characteristics, e.g. enzymatic activity (Sokol et al., 2008), are also important factors for allergy development (Valenta and Kraft, 2004).

The pathophysiology of a type I hypersensitivity reaction (Fig. 1) begins when an allergen that enters the body is taken up by an antigen presenting cell, processed and presented to naïve CD4 T helper (Th) cells that differentiate into Th2 cells and secrete cytokines such as interleukin-4 (IL-4) and IL-13. IL-4 promotes immunoglobulin class switching in B cells to generate IgE antibodies, which are secreted and bind to their high affinity receptor FcεRI that is expressed on the surface of mast cells and basophils. IL-13 also up-regulates IgE synthesis and induces the expression of adhesion molecules and chemokines important in airway inflammation (Wills-Karp, 2004). A second exposure to the allergen then leads to cross-linking of the surface bound IgE antibodies and their FcεRI receptors, which triggers the degranulation of mast cells and basophils releasing mediators such as histamine, leukotriens and prostaglandins. The released mediators cause vasodilatation, mucus secretion, smooth muscle contraction, and can manifest clinically as

allergic rhinitis, asthma, conjunctivitis, and eczema. Massive basophile degranulation manifests as systemic anaphylaxis (Akdis and Blaser, 2000).

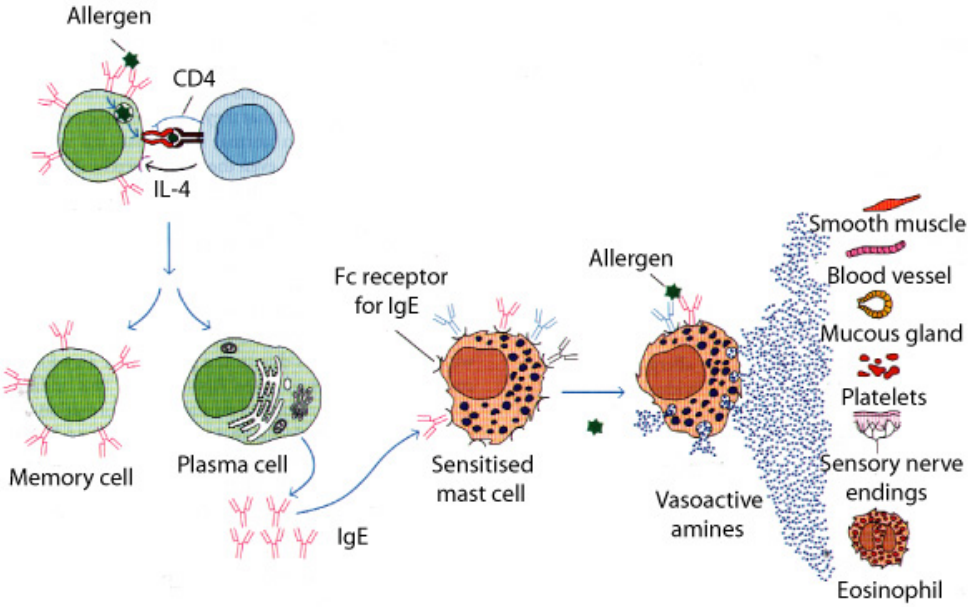

Figure 1. Pathophysiology of an allergic reaction (Goldsby, 2003)**.**

Currently, the only curative treatment of allergy is immune modulation through allergen-specific immunotherapy (SIT). SIT modifies the progression of allergy, and it may also prevent the progression to asthma in patients with allergic rhinitis (Bousquet et al., 1998a; Frankland and Augustin, 1954; Malling, 2004; Wheeler and Woroniecki, 2004). Long-term effectiveness, with persisting relief of symptoms after cessation of treatment, has been demonstrated (Durham et al., 1999b), and SIT is therefore recommended as a first-line therapy for allergies (Bousquet et al., 1998b). However, only a few percent of allergy patients choose to undergo SIT, mainly because the treatment is very time consuming, as it requires 30-70 allergen injections over 3-5 years, and because the allergen injections frequently cause allergic side effects (Casale, 2004).

During SIT, gradually increasing allergen doses are subcutaneously administered until a maintenance dose is reached, and then treatment continues for up to five years. Such lengthy treatment, often requiring as much as 80 allergen injections and frequently causes allergic adverse events. This is why the administered allergen doses must be kept relatively low, which favors Th2 immune responses (Ewbank et al., 2003; Fratil et al., 2006; Haugaard et al., 1993; Incorvaia et al., 2006; Von Garnier et al., 2002).

Although progress has been made in the understanding of the immunological mechanism of SIT, the overall risk/benefit ratio of immunotherapy has not changed over

the last 30 years (Casale, 2004). This may be due to variations in clinical practice, errors in dosing, labeling and patient identification, the absence of standardized extracts for many important allergens or the administration of injections to inappropriate candidates, especially patients with poorly controlled asthma (Bousquet et al., 1998a; Casale, 2004; Passalacqua et al., 2004). Moreover, surveys of healthcare professionals involved in allergy treatment have confirmed that avoidable errors in patient and allergen identification and lack of adequate supervision following immunotherapy injections are largely responsible for treatment-associated fatalities first highlighted by a British report in 1986 (Medicines, 1986). In the US, between 1990 and 2001, a total of 20 deaths were reported, 19 due to immunotherapy and one as a result of skin prick testing (Nelson, 2005). Fortunately, a number of recent developments suggest improvements in both the safety and the efficacy of immunotherapy. These improvements include the use of sublingual immunotherapy (SLIT) (Gidaro et al., 2005; Passalacqua et al., 2004), administration of synthetic peptides (Fellrath et al., 2003; Kay and Larche, 2004), recombinant and engineered allergens (Johansen et al., 2005c; Kussebi et al., 2005; Valenta and Kraft, 2001) and the use of novel adjuvants (Degen et al., 2003; Johansen et al., 2005b). Other new developments include the use of "rush" or "ultra-rush" immunotherapy, by which the primary period of reaching the maintenance dose is shortened. Finally, the recently introduced anti-IgE antibody omalizumab (Rolinck-Werninghaus et al., 2004) (Bousquet et al., 2005; Casale et al., 2006) is presently being used in combination with conventional immunotherapy. Some of these new therapeutic options are reviewed in the context of existing practice.

A SHORT HISTORY OF IMMUNOTHERAPY

In 1819, the London physician John Bostock first described hay fever as a disease that affected the upper respiratory tract in hay-working farmers who showed allergic symptoms such as rhinitis, flushing, and increased temperature (Jackson, 2001). In 1869, Dr. Charles Blakely investigated his own hay fever and performed the first skin test by applying pollen through a small break in his skin (Jackson, 2001). In 1890, he noticed that administering grass pollen to the eyes of patients with hay fever symptoms, today known as the conjunctival provocation test, triggered allergy-like reactions. This was the first demonstration that exposure to pollen was the most probable cause of hay fever. In 1902, Drs. Charles Richet and Paul Portier introduced the word anaphylaxis when in the course of

other immunization research they discovered this life-threatening response to medications and protein substances (Jackson, 2001). Anaphylactic shock occurs within minutes after allergen exposure, causing symptoms such as swelling of body tissues, vomiting, cramps, sudden drop in blood pressure or even a loss of consciousness. It often occurs in people who are highly sensitive to penicillin, stinging insects, shellfish, and nuts and it must be treated as a medical emergency. Four years later, in 1906, the Austrian pediatrician Clemens von Pirquet first used the word allergy to describe the peculiar non-disease related symptoms developed by some diphtheria patients in response to treatment with horse serum antitoxin (Jackson, 2001). The word comes from the Greek word “allo”, meaning, “change in the original state”. Indeed, an allergic reaction is the result of the body's change when it adversely responds to a harmless substance.

Between 1911 and 1914, the work of Leonard Noon and John Freeman at Saint Mary's tuberculosis hospital in London helped to establish the basis for immunotherapy (Freeman, 1911; Freeman, 1914; Noon, 1911). They treated hay fever patients with subcutaneous injections of increasing amounts of aqueous grass pollen extract, starting with very low doses in order to minimize the risk of potentially life-threatening anaphylactic reactions. The maximum dose was administered just before the start of the pollen season. Improvement of symptoms occurred in approximately 75% of the patients. Back then, at a time where IgE was not discovered, Noon and Freeman thought that the pollen-induced symptoms were caused by toxins in the pollen. Being inspired by their colleague Dr. Wright, who at that time was introducing typhoid vaccination in England, Noon and Freeman wanted to use pollen extracts to vaccinate against those toxins. The treatment regime introduced by Noon and Freeman has essentially remained unchanged over the last century. Subcutaneous injections of allergens has remains the preferred SIT method (Wheeler and Woroniecki, 2004).

MECHANISMS OF ALLERGEN-SPECIFIC IMMUNOTHERAPY

SIT has been shown to reduce both symptoms of allergy and the need for supplementary symptomatic medication. In controlled clinical trials, SIT improved the quality of life of treated individuals (Bousquet et al., 1998c; Jayasekera et al., 2007; Till et al., 2004). SIT increases tolerance to allergen challenge, and decreases immediate and late-phase IgE-mediated allergic inflammation. These changes are associated with changes in

cellular and humoral responses to the allergen (Fig. 2). Following SIT, the ratio of Th1 to Th2 cytokines is increased (Bousquet et al., 1998b), and allergen-specific regulatory T cells are induced (Akdis and Blaser, 2000). The production of IL-10 by monocytes, macrophages, B cells and T cells is increased (Akdis et al., 2001; Robinson et al., 2004). The expression of transforming growth factor- β (TGF-β) is increased and, together with IL-10, TGF-β might contribute to regulatory T-cell function and immunoglobulin class switching to IgA, IgG1 and IgG4 (Jeannin et al., 1998; Levings et al., 2002; Nouri-Aria et al., 2004). These immunoglobulins compete with free- or surface bound-IgE for allergen binding (Durham and Till, 1998; Reisinger et al., 2005). This competition decreases allergen capture and presentation that is facilitated by IgE via its high-affinity (FcεRI) or low-affinity receptor (FcεRII) (van Neerven et al., 1999). In the late phase of an IgE mediated allergic reaction, eosinophils, neutrophils and basophils are recruited to the site of inflammation where they perpetuate the inflammatory response by releasing inflammatory mediators. Successful SIT has been shown to reduce the number of mast cells and their ability to release mediators (Durham et al., 1999a). Also the recruitment of eosinophils and neutrophils to sites of allergen exposure is reduced (Varney et al., 1993).

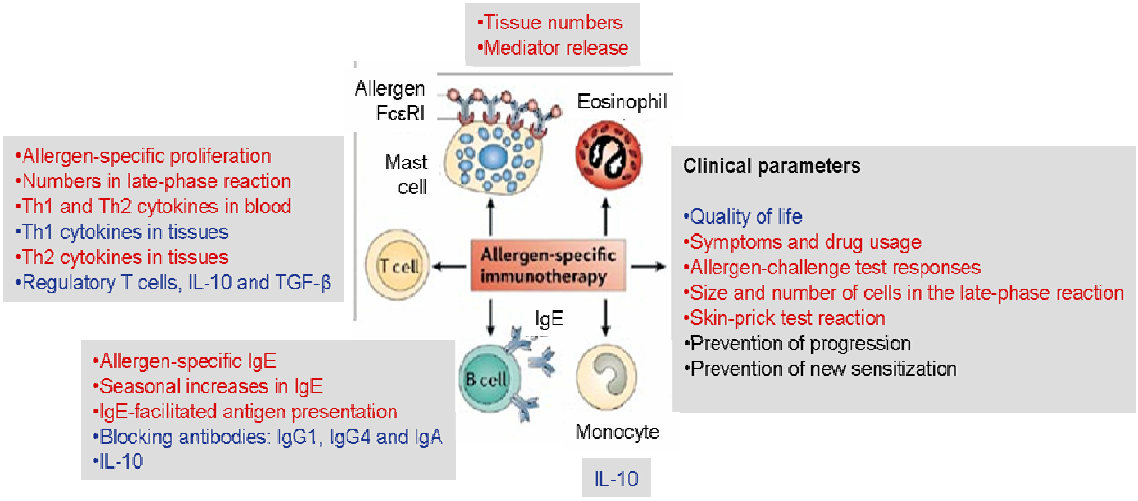

Figure 2. Mechanisms of SIT (Larche et al., 2006). The parameters that increase after SIT are illustrated in blue and the ones that decrease in red.

NOVEL STRATEGIES IN IMMUNOTHERAPY

The major disadvantages of current allergen SIT are its long duration, the high number of injections required and the associated high costs. SIT is also associated with a significant risk of severe allergic reactions. It is therefore of great interest to improve SIT by reducing the number of required injections, and to reduce the side effects by optimizing the allergen type, dose and route of administration. The advantages and disadvantages associated with the strategies used to improve SIT described in this text, are illustrated in Table 1.

Routes of Administration

An important issue in immunotherapy is the route of allergen administration. Interest in alternative routes increased sharply in response to a 1986 report from the British Committee for the Safety of Medicines showing 26 fatalities caused by subcutaneous immunotherapy (Medicines, 1986). Nasal administration has been shown to be effective (Barbey et al., 2004) but its use may be limited by local side-effects, i.e.nasal discharge, blocking and sneezing. In 1999, a Position Paper by a group of experts at the World Health Organization (WHO) concluded that in particular the sublingual route of allergen administration was a viable alternative to the subcutaneous route (Bousquet et al., 1999). More recently, we have suggested that injection of allergens directly into lymph nodes may represent a valuable alternative to conventional SIT (Johansen et al., 2005b; Johansen et al., 2005c).

(i) Subcutaneous immunotherapy

Subcutaneous immunotherapy has been shown to be a highly effective treatment for seasonal allergic rhinitis. A systematic review by WHO experts concluded that immunotherapy was effective in the treatment of allergies to insect venoms and to inhalant allergens, such as pollen, dust mite, cat hair, and mould (Bousquet et al., 1998a). SIT had a beneficial effect on bronchial symptoms and decreased the need for asthma medication in patients with allergic asthma due to pollen. Subcutaneous immunotherapy appears to be more effective in patients with single rather than multiple allergies and more effective in the treatment of pollen-induced seasonal rhino-conjunctivitis than perennial allergy. Long-term exposure to perennial allergens such as dust mite and mould may cause permanent inflammatory changes to airway mucosa and even irreversible remodeling of lung tissue in

patients leading to chronic asthma. In patients with bronchial symptoms to cat allergens, subcutaneous immunotherapy resulted in improvement of symptoms and a reduction in the need for medication. After many years of debate, a landmark study, which revealed that subcutaneous immunotherapy against grass pollen allergy lead to clinical remission and persisting reduction in immunological reactivity, confirmed the long-term efficacy of SIT (Durham et al., 1999b).

High efficacy of subcutaneous immunotherapy is observed in insect venom allergic patients. The efficacies are typically 80-95%, and such *Hymenoptera*-venom immunotherapy represents the only effective prophylactic treatment for insect sting-induced anaphylaxis (Bousquet et al., 1998a; Casale, 2004; Golden, 2005). SIT is, therefore, recommended as the first line therapy for patients with a history of severe systemic allergic reactions to insect stings. After three to five years of immunotherapy most patients remain protected against subsequent stings, even if they remain skin test positive (Bousquet et al., 1998a). Only 25% of patients become skin-test negative after 3-5 years of insect venom SIT and after 7-10 years 60–70% of patients become skin test negative (Golden, 2005). If patients become skin test negative, it is considered safe to discontinue venom subcutaneous immunotherapy (Bousquet et al., 1998a).

(ii) Sublingual immunotherapy

In sublingual immunotherapy (SLIT), the allergen is given as tablets or as drops that are kept under the tongue for a few minutes (Gidaro et al., 2005; Passalacqua et al., 2004). Although a WHO Position Paper in 1998 stated that only four studies had by then demonstrated the clinical effectiveness of sublingual-swallow immunotherapy (Bousquet et al., 1998a; Malling et al., 1998), its efficacy has been assessed in a number of studies in recent years. A further advantage of SLIT is that it enhances compliance, likely due to both the low rate of side-effects (Nelson, 2005) and the needle-free administration at home.

A meta-analysis of 22 randomised double-blind, placebo-controlled trials involving 979 patients with allergic rhinitis caused by house dust mite, grass pollen, *Parietaria*, olive and ragweed allergens (Wilson et al., 2005) revealed significant reductions in both rhinitis symptoms and medication requirements following SLIT. Moreover, the direct comparison of subcutaneous SIT and SLIT showed similar improvements both in symptoms and medication requirements for the two routes of administration (Wilson et al., 2005). However, it should be noted that the allergen doses used in recent SLIT studies were from

3–500 times higher than those used in subcutaneous immunotherapy (Gidaro et al., 2005; Malling, 2004; Wilson et al., 2005).

SLIT has also been shown to reduce symptoms and use of β_2-agonists and systemic steroids in patients with allergic asthma (Bousquet et al., 1999; Passalacqua et al., 2004; Purello-D'Ambrosio et al., 1999). Improvements in lung function, bronchial hyper-reactivity and quality of life have also been noted (Bousquet et al., 1999; Lombardi et al., 2001). At least one study indicated that the efficacy of SLIT in allergic asthma is dose dependent (Andre et al., 2003). Despite the limited data available to assess the long-lasting effect of SLIT in asthma, a recent study performed in 35 children treated for 4–5 years with SLIT indicated a significant improvement of asthma, as long as five years after discontinuation of treatment (Di Rienzo et al., 2003; Passalacqua et al., 2004).

Current research to improve SLIT focuses on optimizing the allergen molecules and the adjuvants used. For instance, the intranasal administration of recombinant Bet v 1, the major birch pollen allergen, was shown to reduce systemic allergic immune responses and airway inflammation in birch pollen sensitized mice (Winkler et al., 2002). In the same way, oral administration of a T-cell epitope peptide from the major Japanese cedar, allergen Cry j 2, induced immunological tolerance in previously sensitized mice (Yoshitomi et al., 2007). Chimeric allergens composed of the recombinant Bet v 1 allergen linked to two peptides derived from the major grass pollen allergens Phl p 1 and Phl p 5, induced mucosal tolerance when used prior to poly-sensitization and were suggested to prevent multi-sensitizations (Wild et al., 2007). Alternatively, the modification of allergens with mucoadhesives, e.g. maltodextrin, not only enhanced mucosal adhesion of the allergen, but also induced tolerance by reducing airway hyper-responsiveness, airway inflammation and IL-5 production in mice, which consequently increased the efficacy of SLIT (Razafindratsita et al., 2007). Furthermore, several studies have used adjuvants such as polymeric microparticles to increase the immunogenicity of encapsulated antigens for oral and intranasal administration in mice (O'Hagan, 1998; Roth-Walter et al., 2005; Vajdy and O'Hagan, 2001). In a recent study in mice, intranasal administration of an allergenic peptide encapsulated in PLGA microparticles prevented subsequent systemic allergic immune responses and airway inflammation to the whole allergen (Marazuela et al., 2008). Additionally, the use of microparticles containing timothy grass pollen extract for oral immunization in allergic patients has been shown to reduce effectively medication and symptoms scores (TePas et al., 2004).

(iii) Other routes of administration

In mice, the immunogenicity of antigens administered directly into a subcutaneous lymph node (intranodal administration) has been shown to be superior to the conventional subcutaneous administration (Johansen et al., 2005a; Maloy et al., 2001). Such intralymphatic immunization triggers Th1-like immune responses with enhanced secretion of IgG2a antibodies and reduced IgE production. In contrast, subcutaneous immunization with allergens induces Th2-associated IgG1 and IgE antibodies and little IgG2a. Intranodal injections require lower allergen doses than subcutaneous administration. This also reduces the risk of allergic reactions in SIT (Martínez Gómez et al, paper submitted). The first phase I/IIa clinical trials using the intranodal route of administration to desensitize allergic patients has revealed promising results. The results from a double-blinded phase I/II trial on grass and tree pollen allergic patients suggested that the efficacy of eight weeks with intranodal immunotherapy was comparable to three years of immunotherapy with conventional subcutaneous injections (Senti et al, paper submitted). Hence, intranodal immunotherapy may be a promising alternative to subcutaneous SIT not only in terms of efficacy and safety, but also in regard to compliance, since the treatment time can be strongly reduced.

Allergens

In the US the original type of immunotherapeutic aqueous allergen extracts, which contain a mixture of molecules from pollen, mould, dust mite or other allergenic substances, are still widely used, despite the risk of allergic side effects upon administration. In Europe, purified and modified allergens and adjuvants forming a slow release depot are used to reduce this risk. To produce purified extracts, unwanted low molecular weight material is removed, as most true allergens have a molecular weight > 10 kDa (Wheeler and Woroniecki, 2004).

One strategy to increase safety and tolerability of SIT is to inject the allergen in a form that is not recognized by IgE. In the 1970-80s allergens were chemically modified by using formaldehyde, glutaraldehyde, tyrosine, alginates to form so-called allergoids (Bousquet et al., 1998a; Degen et al., 2003; Patterson et al., 1977; Wheeler and Woroniecki, 2004). These methods not only reduced reactogenicity with IgE, but also improved antigen processing, Th1 cytokine secretion, and therapeutic efficacy (Bousquet et al., 1990; Gieni et al., 1996; Grammer et al., 1987). More recently, naked DNA vaccines expressing an

allergen have been utilized (Jilek et al., 2001; Jilek et al., 2004). Recombinant DNA technology has also facilitated the production of mutated allergens with low IgE-binding capacity (Ferreira et al., 1998; Kussebi et al., 2005; Schmid-Grendelmeier et al., 2003; Tresch et al., 2003; Valenta and Kraft, 2002; Westritschnig et al., 2004), allowing the use of higher therapeutic doses (Andre et al., 2003; Ewbank et al., 2003). For the same reason, short, synthetic peptide sequences corresponding to T-cell epitopes from the allergen which but do not bind IgE have been used for SIT (Alexander et al., 2002; Kay, 2004; von Garnier et al., 2000).

(i) Recombinant allergens

Advances in molecular biology have allowed the cloning of most common allergens and the investigation of their nature (Valenta and Kraft, 2002). This has allowed production of defined allergens, or recombinant molecules with improved immunological properties derived from the original allergens. The technology has opened new avenues in diagnostics and in immunotherapy (Chapman et al., 2000; Ferreira et al., 2002; Valenta and Kraft, 2004; Valenta and Niederberger, 2007).

Wild-type recombinant allergens mimic the immunological and structural properties of natural allergens. They can replace allergen extracts, offering the advantage that defined molecules in defined quantities and quality can be formulated as vaccines. Wild-type allergens have been used in animal models to induce blocking antibodies (de Weerd et al., 2003; Swoboda et al., 2004) and tolerance (Hufnagl et al., 2003). Clinical efficacy has been demonstrated n a randomized, double-bind, placebo controlled trial using several recombinant grass pollen allergens for SIT. (Jutel et al., 2005). Alternatively, the covalent linking of several major allergens to form hybrid allergen molecules was found to increase the immunogenicity of the individual components and, hence, to induce higher levels of blocking antibodies in animals than vaccination with a mix of the allergens (Kussebi et al., 2005; Linhart et al., 2005). Although the treatment with wild type-like allergens offers advantages with respect to pharmaceutical quality, standardization and dosage formulation of the vaccine, so far the duration of the treatment remains similar to SIT with allergen extracts.

Recombinant technology has also paved the way for modified allergens or so-called bioengineered muteins (Bousquet et al., 1998a; Devos et al., 1995; Wheeler and Woroniecki, 2004). Muteins are modified allergen molecules that retain the ability to

stimulate Th1 responses but do not react with allergen-specific IgE antibodies (Bhalla et al., 2001; Swoboda et al., 2002). They are derived from allergenic proteins by replacing of one or more amino acids from their IgE antibody binding sites. For instance, by mapping sequential epitopes on the major shrimp allergen, tropomyosin (Pen a 1), a mutant with strongly reduced allergenicity but with potential therapeutic use was generated (Reese et al., 2005). We have developed and tested recombinant fusion proteins carrying additional immunological targeting sequences, such as the so-called Modular Antigen Transporter (MAT)-allergens (Fig. 3) (Crameri et al., 2007). These allergens enable the rapid translocation of the allergen into antigen-presenting cells (through a Tat sequence derived from the human immunodeficiency virus) and targeting to the MHC class-II presentation pathway (through a truncated human invariant chain, li). We showed that MAT-Fel d 1 allergen increased therapeutic immunogenicity of Fel d 1 in mice, inducing high levels of IgG2a and IFN-γ, while reducing IL-4 secretion by allergen specific T cells. At the same time MAT-Fel d 1 showed reduced degranulation of human basophils as compared to wild-type recombinant allergen, suggesting reduced binding of surface IgE (Martínez Gómez et al. paper submitted).

Likewise, a novel Fcγ-Fel d 1 bifunctional fusion protein was shown to inhibit FcεRI-mediated degranulation in a dose-dependent manner by direct cross-linking of anti-Fel d 1 IgE bound FcεRI (IgE high affinity receptor) and FcγRIIb (receptor containing inhibitory motifs) on the surface human mast cells and basophils (Zhu et al., 2005, Zhang, 2007 #1208). Immunization of Fel d 1-sensitized mice with Fcγ-Fel d 1 has been shown to block systemic allergic reactivity. The inhibition of histamine release from mast cells and basophils of cat allergic donors was associated with altered Syk and ERK signaling (Terada et al., 2006).

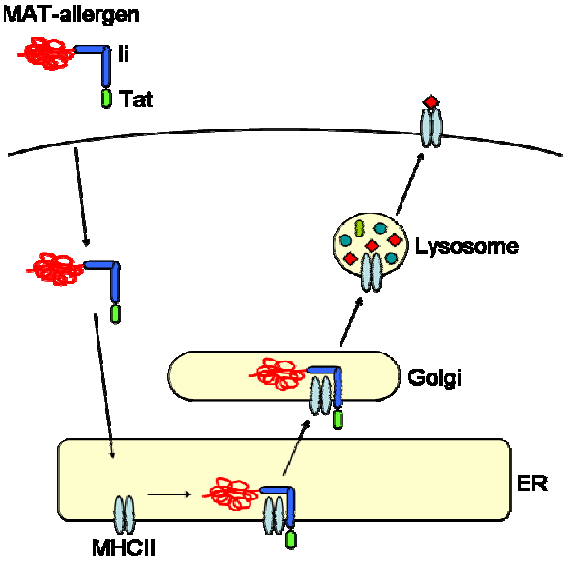

Figure 3. Proposed mechanism for an increased antigen presentation through the MHC-II presentation pathway. Modular antigen translocation (MAT) vaccines consist of a translocating peptide (green) converting extracellular to cytoplasmic proteins, a domain derived from the invariant chain Ii (blue) targeting the fusion protein to the endoplasmatic reticulum where assembly of MHC-II and Ii complexes occurs, and an allergen of interest fused C-terminally to Ii (red) (Crameri et al., 2007).

The safety benefits of recombinant and engineered allergens remain to be fully determined, but early results with a genetically modified Bet v 1 allergen demonstrate its immunotherapeutic efficacy (Niederberger et al., 2004; Niederberger et al., 2007). However, the theoretical advantages of such molecules may be outweighed by high production costs and the pharmaceutical development of such molecules as pharmaceutical products. As most allergies are caused by multiple allergens, e.g. hay fever and house dust mite allergy, a vaccine would have to consist of numerous different recombinant allergen molecules. In this respect, an alternative and easier non-recombinant method to reduce IgE-binding capacity has been achieved by physical disruption of the secondary and tertiary structure of protein allergens by heat-denaturation (Koppelman et al., 2002; Magi et al., 2004). The use of heat-denaturation is in line with the clinical observations that allergic individuals usually tolerate cooked food better than raw food (Sanchez-Monge et al., 2000). In mice, heat-denatured allergens showed a lower sensitization potential but enhanced IgG2a antibody- and Th1-inducing capacity, showing the potential of such allergens for use in SIT (Johansen et al., 2005c).

(ii) Peptides

The use of short immunodominant peptides derived from allergens have been shown to decrease the risk of anaphylaxis inherent to allergen-specific immunotherapy (von Garnier

et al., 2000). Therapeutic peptides typically contain T-cell epitopes that trigger T-cell immune responses, but lack IgE reactivity (Fellrath et al., 2003; Kay and Larche, 2004; Wheeler and Woroniecki, 2004). This approach was successfully applied with restricted sets of immunodominant peptides derived from Fel d 1, bee venom phospholipase A2 (Api m 1) (Fellrath et al., 2003; Muller et al., 1998; Norman et al., 1996; Von Garnier et al., 2002; von Garnier et al., 2000), or Bet v 1 in mice (Bauer et al., 1997; Briner et al., 1993; Oshiba et al., 1996). A mixture of three short peptides that stimulated T-cell clones from allergic and non-allergic study subjects were used in patients allergic to bee venom (Muller et al., 1998). These subjects subsequently tolerated an injection of Api m 1 equivalent to one bee sting and some subjects were able to tolerate a bee sting. Similarly, in studies using the peptides derived from Fel d 1, efficacy was demonstrated, but the FDA put further trials on hold, as several hours after the peptide injection a T cell mediated late phase asthma response was observed (Norman, 2004; Wheeler and Woroniecki, 2004). Intranasal administration of the immunodominant house dust mite allergen Der p 1 in primed mice reduced T-cell responses not only to the epitope, but also to the entire protein via so-called linked immunosuppression (Hoyne et al., 1993). However, though these studies were promising, contradicting data were also published. Spertini and co-workers showed that intranasal therapy with ovalbumin (OVA) but not with its major T-cell epitope led to T-cell activation and the induction of IL-10 secreting CD4 T-cells in the bronchial lymph nodes (Barbey et al., 2004). Subcutaneous immunization with the major T-cell epitope-containing peptide of the house-dust mite Der p 2 elicited a strong immune response rather than a state of tolerance (Wu et al., 2000). In a murine model of asthma, immunotherapy with OVA reduced airway eosinophilia and hyper-responsiveness, whereas immunotherapy with the immunodominant OVA peptide 323–339 enhanced them (Janssen et al., 1999).

Although peptides that do not bind IgE might represent a good alternative to wild-type allergens, they do not strongly induce blocking IgG antibodies, and hence, they may not be appropriate for SIT. More research is required to define the epitopes and the sequences that on the one hand do not bind IgE and on the other hand induce T- and B-cell responses that lead to desensitization or tolerance. While mouse studies used inbred strains with defined MHC class II molecules, another obstacle to clinical use is the HLA variation within the human population.

Adjuvants

Adjuvants are defined as a group of structurally heterogeneous compounds, used to evoke or increase immune responses to antigens (Gupta et al., 1993). Classical adjuvants include oil emulsions, saponins, aluminum or calcium salts, non-ionic block polymer surfactants, and derivatives of mycobacteria, lipopolysaccharides, and other bacterial or viral components (Marciani, 2003; O'Hagan and Valiante, 2003; Villinger, 2003). Although the structural requirements of effective adjuvants as well as the molecular and cellular mechanisms behind effective immune responses are not yet completely understood, they have been classified according to five recently proposed concepts of immunogenicity (Schijns, 2000): i) the geographical concept of immune reactivity; ii) the theory of depot effect; iii) the paradigm that adjuvants act as Signal 0 characterized by pathogen recognition through PAMPS (Janeway, 1989); iv) the role of Signal 2 molecules as natural adjuvants in the activation of naive T-helper cells; v) the hypothesis that immunity is activated by exogenous and eventually endogenous danger (Matzinger, 2002).

The adjuvants used in SIT have typically been aluminum and calcium salts. Apart from causing local granuloma formation at the injection sites, these adjuvants have a good safety record (Gupta, 1998). Major disadvantages of aluminum hydroxide are the unpredictable efficacy of adsorption of certain allergens/allergen extracts and stability of the adsorbates, the possibility that allergens are altered in the course of the undefined adsorption process and the difficulties in assessing quality and quantity of allergens once they are adsorbed. Furthermore, they produce poor T-cell responses, triggering preferentially Th2 responses (including IgE antibodies) rather than the therapeutically more favorable Th1 responses. Therefore, an important part of the research to improve SIT now focuses on the use of alternative adjuvants that trigger innate immunity to counterbalance the Th2 immune responses (Vandenbulcke et al., 2006; Yang et al., 2006).

(i) Toll-like receptor ligands

The theory known as the *hygiene hypothesis* suggests that diminished exposure to early childhood infections and environmental bacterial components has resulted in a population that is predisposed to allergies and has, therefore, been suggested as an explanation for the rising prevalence of allergic disorders (Ponsonby and Kemp, 2008; Yazdanbakhsh et al., 2002). A number of studies support an inverse association between early infectious exposures and the development of allergic diseases (Illi et al., 2001; Matricardi et al.,

2002). Several microbial components, which are recognized by pattern-recognition receptors such as Toll-like receptors (TLRs) can directly activate immune cells (Akira et al., 2006; Kaisho and Akira, 2006). Thus, the reduced microbial stimulation of the TLRs in early life, which could lead to a weaker Th1 response and a stronger Th2 response to allergens plays a key role in the *hygiene hypothesis* to. It is believed that compounds that can control the over-expression of Th2 cytokine-secreting cells or skew the Th2-Th1 balance towards the latter would be of clinical advantage for allergy vaccination (Chisholm et al., 2004; Gangloff and Guenounou, 2004). In this respect the use of TLR agonist as adjuvants has opened up new avenues for SIT (Akdis et al., 2003; Degen et al., 2003; Johansen et al., 2005b).

Lipoproteins and lipoteichoic acid (LTA) from Gram-positive bacteria are recognized as a pathogen-associated potential threat by TLR2, which is localized on the surface of immune cells, especially DCs. The stimulation of the innate immune system by the synthetic lipopeptide LP40, which binds to TLR2 and signals through MyD88, induces IL-10, IL-12 and IFN-γ, and inhibits Th2 cells and IgE production in human PBMCs and also lung eosinophilia in several mice models (Cezmi A. Akdis, 2003). Another TLR2 agonist, the synthetic triacylated lipopeptide Pam3CSK4, was shown to attenuate OVA-induced asthma in mice. This attenuation of asthma was also associated with increased IL-12, IFN-γ and IL-10 production (Patel et al., 2005). Similarly, the Gram-positive bacteria, *Lactobacillus plantarum,* was shown to modulate allergen-specific responses both *in vitro* and *in vivo* after its co-administration with Der p 1. Increased Th1-associate cytokines, IL-12 and IFN-γ, were observed as well as increased secretion of IgG2a antibodies, and reduced eosinophilia. Again the prevention of allergic responses was modulated by TLR2 and MyD88 (Hisbergues et al., 2007). A different study showed that *L. plantarum* induced IL-10 gene expression in DCs and naïve CD4 T cells, and enhanced the efficacy of sublingual immunotherapy in a murine asthma model (Van Overtvelt et al., 2008).

Several mycobacterial products have been found to activate TLR2 and TLR4 (Heldwein and Fenton, 2002), and to induce strong IFN-γ responses (Walker et al., 2003). Pre-treatment of asthmatic mice with the heat-killed *Mycobacterium vaccae* conferred long-term antigen-specific protection from OVA-induced airway inflammation (Zuany-Amorim et al., 2002). In humans a double-blind trial evaluated the potential of heat-killed *M. vaccae* in patients with atopic asthma (Camporota et al., 2003). Participants were given a bronchial allergen challenge before and after treatment and their early and late asthmatic

responses were monitored. However, despite reduction in the late asthmatic responses, the results were not significantly different from the placebo group.

Lipopolysaccharide (LPS) is a cell-wall component of Gram-negative bacteria that binds to TLR4. The role of LPS and its receptor in allergies is well known (Williams et al., 2005). It has been shown that both the amount and the time of the exposure to LPS determine the type of immune responses elicit following LPS administration. Inhalation of low levels of LPS triggered Th2 responses, whereas high LPS levels resulted in Th1 responses (Eisenbarth et al., 2002). Exposure to LPS early in the sensitization process prevented the development of allergies. In contrast, later exposure aggravated the inflammatory reaction (Tulic et al., 2000). Monophosphoryl lipid A (MPL), a derivative from the LPS of *Salmonella minnesota* that maintains its pro-inflammatory properties but with a lower toxicity, has been shown to induce Th1-skewed immune responses (Wheeler et al., 2001). Several clinical trials have already evaluated the potential of using MPLs in humans (Drachenberg et al., 2001; Mothes et al., 2003). Four pre-seasonal injections of MPL-adjuvanted grass pollen allergoid successfully improved symptoms and medication scores. These effects were associated with high titers of allergen-specific IgG1 and IgG4 antibodies, which could block allergen-dependent degranulation of basophils (Mothes et al., 2003). Despite positive initial results the FDA has stopped phase III trials because of safety concerns (http://www.biospace.com/news_story.aspx?NewsEntityId=62636). Furthermore, two synthetic triacylated pseudo-dipeptides agonists of TLR4 have been described to induce human DC maturation and polarization of naïve T cells towards IFN-γ producing Th1 cells (Mascarell et al., 2007). However, only one of the molecules, OM-294-BA-MP, was able to induce IL-10 gene expression and enhance tolerance after sublingual administration in mice with established asthma to OVA.

The genetic material of microbes has an excellent immune stimulating capacity. Indeed, viral double- and single-stranded RNA is recognized by the endosomal receptors TLR3 and TLR7/8, respectively. A synthetic compound, R-848, also called resiquimod, from the imidazoquinoline family that binds to TLR7 and TLR8 in humans, was shown to shift the differentiation of allergen-specific human CD4 T cells *in vitro* from a dominant Th2-like response to a prevalent Th1-like response. This strong polarizing capacity was attributed to the effect of R-848 on cells from the innate immune system, such as macrophages, natural killer cells and DCs (Brugnolo et al., 2003). Similarly, a study in mice showed that a single intranasal injection of R-848 in OVA-sensitized mice effectively inhibited allergen-induced airway inflammation and hyper-reactivity by reduction of Th2 responses and increased

production of IL-12 and IFN-γ (Quarcoo et al., 2004). Polyinosinic:polycytidylic acid (PolyI:C) a synthetic double-stranded RNA agonist of TLR3 prevented and suppressed asthma in mice as a consequence of the additive effects of IL-10 and IL-12 (Sel et al., 2007).

TLR9 is another endosomal receptor that detects unmethylated CpG-rich sequences relatively common in the genomes of most bacteria and DNA-viruses. In humans, TLR9 is mainly expressed in B cells and plasmacytoid DCs, whereas in mice TLR9 is also expressed in monocytes and myeloid DCs (Iwasaki and Medzhitov, 2004). It has been shown that CpG oligodeoxinucleotides trigger direct B cell activation (Krieg et al., 1995) and that TLR9 can regulate isotype switching to IgG2a not only through activation via Th1 cells (Krieg, 2006) but also by direct interaction with B cells (Jegerlehner et al., 2007). The immunomodulatory effect of CpG motifs can suppress IgE responses induced by both protein and plasmid DNA allergens (Peng et al., 2004), redirect the allergic Th2 responses towards Th1 responses and prevent the development of airway inflammation in mice both prophylactically and therapeutically (Banerjee et al., 2004; Jahn-Schmid et al., 1999; Jain et al., 2003). Furthermore, the conjugation of Amb a 1, a ragweed pollen major allergen, to CpG motifs led to reduced airway hyper-responsiveness in ragweed-sensitized mice. This effect was associated with increased IFN-γ and IgG2a levels (Santeliz et al., 2002). A recent study has shown that immunotherapy with CpG DNA conjugated with a T-cell peptide from the Japanese cedar pollen major allergen Cry j 2, was also useful in preventing and treating allergic conditions (Suzuki et al., 2007).

These promising pre-clinical results have led to several clinical trials. The use of Amb a 1 linked to CpG (AIC) to stimulate PBMCs of ragweed allergic patients induced Th1 responses instead of the Th2 responses.. The treatment of ragweed allergic subjects with escalating doses of AIC was found to reduce *ex vivo* ragweed-specific Th2 responses (IL-5, CCL17, and CCL22), and a transient increase of IFN-γ, CXCL9, and IL-10 was observed (Simons et al., 2004). In two separate studies with six escalating doses of AIC, reduced nasal symptoms as compared to placebo treatment were observed (Creticos et al., 2006; Tulic et al., 2004). Moreover, the protective effect against ragweed-induced symptoms was still observed during the second ragweed season and no increase in serious adverse events was reported. However, the phase III studies have just recently been suspended, as they did not meet their end points (http://www.apmhealtheurope.com/depechesPublieesDepeches.php?annee=2007&mois=2&jour=27).

The risk of rapid degradation of CpG by nucleases together with the fact that TLR9 is an intracellular receptor has made the idea of its encapsulation in particulate systems such as liposomes (Joseph et al., 2002), VLPs (Storni et al., 2004) and PLGA (Hunter et al., 2001; Martinez Gomez et al., 2007) especially attractive. Some of these systems have been evaluated in allergy vaccines and will be described in the next section.

(ii) Particles

Particles have been subject to intense investigation with regard to their adjuvant potential, in part, because the immune system has evolved to fight microorganisms, which mostly have a particulate nature. Therefore, it is not surprising that particulate antigens in the size range of pathogens (Fig. 4a) can act as adjuvants by direct targeting of antigen presenting cells (O'Hagan and Valiante, 2003; Storni et al., 2005). Typically, particles are well recognized by the innate immune system (Fig. 4b) as foreign or as danger, hence, triggering the onset of an immune response. The combination of aluminum hydroxide and allergens, introduced for depot vaccination 70 years ago (Sledge, 1938), also consists of suspended aggregates or particles and thereby improve immune-stimulatory properties of allergens. However, in contrast to infectious particles that induce strong cell-mediated immune responses including strong Th1 responses (Storni et al., 2005), aluminum-based allergy vaccines typically give rise to Th2 responses and are sub-optimal for immunotherapy. Therefore, novel adjuvant particles with stronger Th1-triggering properties would be advantageous.

Biodegradable polymers

Among the most studied polymers for drug or antigen delivery is poly(lactide-co-glycolide) (PLGA), which is biocompatible and approved by the Food and Drug Administration (FDA) for use in humans. There are two main properties of polymeric microparticles that make them particularly interesting for SIT. Firstly, they have the capacity to sustain the delivery of drugs or antigens (Varde and Pack, 2004). The time period over which this delivery is to be sustained can be controlled by using polymers with different physico-chemical characteristics, e.g. molecular weight and the relative relationship of lactide and glycolide (Johansen et al., 2000b). Secondly, and as discussed above, the particulate nature of microparticles provides a mechanism to deliver antigens efficiently to the lymphatic system, while protecting them from degradation in the extracellular fluids. A wide variety of proteins, peptides and even plasmid DNA have been

successfully encapsulated into PLGA particles, and they have been shown to induce protective immunity after a single subcutaneous injection (Peyre et al., 2004; Peyre et al., 2003). Additional features of the microparticles such as their size (Gutierro et al., 2002) and surface properties (Wischke et al., 2006) (Martínez Gómez et al., paper submitted) can be modified to influence the type of immune response.

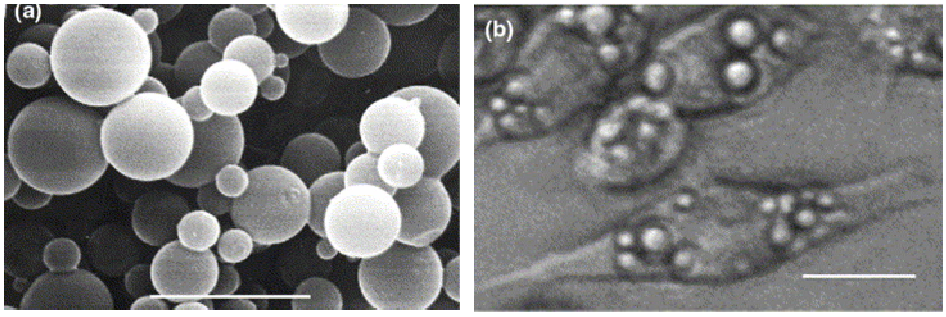

Figure 4. (a) Poly (lactide-co-glycolide) microspheres photographed by scanning electron microscopy (bar=10 μm). Photograph courtesy of E. Wehrli, ETH Zürich (Zürich, Switzerland). (b) *In vitro* cultivated macrophages (RAW) that have engulfed microspheres of 2–5 μm size (bar=10μm). Photograph courtesy of Y. Men, ETH Zürich (Johansen et al., 2000a).

Over the last years encapsulation of several allergens into PLGA micro/nanoparticles has been described. Encapsulation of Ole e 1, the major olive pollen allergen, showed no detrimental effect on the integrity and antigenicity of the allergen (Batanero et al., 2002). Moreover, immunization of mice with encapsulated Ole e 1 enhanced Th1-like responses as compared to Ole e 1 adsorbed on alum (Batanero et al., 2003). A recent study in mice showed that the prophylactic intranasal administration of PLGA microparticles containing a dominant T-cell epitope of Ole e 1 prevented sensitization to the whole protein. Mice had higher IgG2a levels and lower IgG1 and IgE as well as reduced IL-4 and IL-10 compared to the mice treated with empty particles. Furthermore, the airway inflammation induced after challenge with Ole e 1 could be attenuated after pre-treatment with the allergen-loaded particles (Marazuela et al., 2008).

Another prophylactic study showed that administration of PLGA loaded with plasmid DNA encoding for the bee venom allergen phospholipase A2 (PLA2) prior to sensitization protected mice against anaphylaxis. To explain the tolerance observed in sensitized mice after PLGA injection, the authors proposed a dual mechanism of action that involved initially an immune deviation from Th2 to Th1 and then IL-10-mediated suppression (Jilek et al., 2004).

Schöll et al. were the first to describe the therapeutic potential of PLGA nanoparticles in a murine model of allergy. After a single subcutaneous injection of Bet v 1 encapsulated

in PLGA nanoparticles, the ongoing Th2 responses observed in sensitized mice were re-directed towards Th1. This down-regulation of Th2 responses was associated with high IgG2a titers and increased IFN-γ and IL-10 production (Scholl et al., 2004).

The surface of microparticles can be modified to target particular cell types or tissues. For instance, the functionalization of particles with lectins, which bind to specific carbohydrate moieties on the glycocalyx of cellular membranes, have been used for oral delivery and mucosal targeting of allergens (Gabor et al., 2004; Lavelle, 2006). Bet v 1 loaded PLGA microparticles have been covalently linked to wheat germ agglutinin (Walter et al., 2004) or *Aleuria aurantia* lectin (Roth-Walter et al., 2005). Such particles did not only protect the allergen from rapid degradation in the gastrointestinal tract but they also prolonged antigen release at the intestinal site and induced stronger allergen-specific Th1 responses than uncoated particles or the naked allergen.

Alternatively, the co-encapsulation of antigens and immunostimolatory agents has been shown to modulate immune responses (Antje Heit, 2007; Kazzaz et al., 2006). In this respect, we showed that PLGA microparticles with co-encapsulated PLA2 and CpG were able to induce long-lasting Th1 immune responses. Moreover, bee venom-sensitized mice treated with co-encapsulated PLA2 and CpG microparticles had higher IgG2a levels, which correlated with a better protection against anaphylaxis than mice treated with the encapsulated PLA2 alone or with PLA2-PLGA and admixed CpG prior to injection. The use of protamine to stabilize the CpG was found to further enhance the immunogenicity of the PLA2 and caused a more pronounced protection against anaphylaxis than when CpG was not stabilized (Martinez Gomez et al., 2007).

Microparticles fabricated from poly-ε-caprolactone (PCL), showed slower degradation rates than PLGA particles and did not generate an acidic micro-environment, which could damage encapsulated antigens (Lemoine et al., 1996). However, so far only one study has evaluated the potential of PCL particles for allergy vaccines. In OVA-sensitized mice, treatment with OVA in PCL particles or adsorbed to aluminum salts induced similar levels of IgG but aluminum induced higher IgE levels. Consequently, mice treated with OVA in PCL had reduced histamine secretion and body temperature drop after a challenge with a high dose of OVA (Roman et al., 2007).

The synthetic copolymer poly(methyl vinyl ether-*co*-maleic anhydride) (PMVE/MAH, which is commercially branded as Gantrez® AN) is widely used for pharmaceutical purposes such as a thickening and suspending agent, a denture adhesive and as adjuvant for transdermal patches (Arbos et al., 2002). PMVE/MAH is a biodegradable polyanhydride,

which could be an appropriate copolymer to simplify the preparation of ligand–nanoparticle conjugates and for the preparation of particulate dosage forms with bioadhesive or mucoadhesive properties. The potential of Gantrez® nanoparticles as an adjuvant for oral allergen immunotherapy has been evaluated in a mouse model of allergy (Gomez et al., 2007). Increased levels of both IgG1 and IgG2a were found in naïve mice after the oral administration of OVA-entrapped nanoparticles as compared to OVA in solution. The same study confirmed the potential of Gantrez® nanoparticles to protect mice from anaphylaxis in a therapeutic allergy model. OVA-sensitized mice treated with the OVA-entrapped nanoparticles were 100% protected against mortality in a challenge with a high dose of OVA, whereas only 40% of mice treated with OVA in solution were protected.

Carbohydrate-based particles

Three clinical studies in the mid-1990s evaluated the capacity of particles made of a sugar-starch core, coated with ragweed allergen extract, and then enterocoated with methacrylic acid copolymer, for oral administration (Litwin et al., 1996; Litwin et al., 1997; Van Deusen et al., 1997). The patients that could tolerate a high dose of the encapsulated allergen extract had significantly decreased allergic symptoms, developed high titers of ragweed IgG, had an initial increased of IgE, down-regulated the expected seasonal increase in nasal specific IgA, and had decreased nasal sensitivity after a provocation test. Despite the encouraging results, more than ten years have passed without any further studies published using such particles.

Chitin is the second most abundant natural polysaccharide. It consists of N-acetyl-D-glucosamine units, and is the major component of the cell wall of fungi and the exoskeleton of crustaceans and insects. Its derivative chitosan is commercially obtained by deacetylation of chitin. It is biocompatible, biodegradable and bioadhesive, it readily binds to negatively charged surfaces like those of mucosal membranes, enhancing the transport of polar drugs across epithelial surfaces (Singla and Chawla, 2001).

Chitin-based particles of 1-10 μm size can be phagocytosed by macrophages through the mannose receptor, and are potent stimulators that promote Th1 immune responses (Shibata et al., 1997). Orally administered chitin particles down-regulated serum IgE and lung eosinophilia in a mouse model of ragweed allergy (Shibata et al., 2000). Another study revealed down-regulation of allergic responses to *Aspergillus fumigatus* and *Dermatophagoides pteronyssinus* after intranasal administration of chitin microparticles.

Elevated intracellular levels of IFN-γ and TNF-α and reduced IL-4 during allergen challenge were found, suggesting a modulation of the immune response from Th2 to Th1 (Strong et al., 2002). This beneficial effect of chitin particles was also observed in asthmatic mice (Ozdemir et al., 2006).

Chitosan particles do not induce by themselves Th1 cytokines in the way chitin particles do, but they can complex DNA and effectively protect it from degradation (Borchard, 2001; Mansouri et al., 2004; Richardson et al., 1999). This property has been successfully exploited in pre-clinical studies. The complex of chitosan and peanut or house dust mite allergen-plasmid DNA induced immunological protection in murine models of peanut (Roy et al., 1999) and house dust mite (Chew et al., 2003) allergies. In addition, prophylactic as well as therapeutic treatment of asthmatic mice with plasmid IFN-γ complexed to chitosan nanoparticles resulted in effective reduction of allergen-induced airway inflammation and airway hyper-responsiveness. It was suggested that STAT4 signaling was involved in the mechanism of action of chitosan particles (Kumar et al., 2003).

Carbohydrate-based particles (CBP) made of sepharose with a size diameter of 2 μm, have been proposed as an alternative to aluminum salts as adjuvant for SIT. Allergens can be covalently coupled with a high density without altering their immunological properties via cyanogen bromide activation of the particles. Phl p 5b, a major timothy grass pollen allergen, coupled to CBP was tested in mice and showed elevated IFN-γ production and IgG2a/b levels, indicating a polarized Th1 response and did not cause granulomatous tissue reactions as compared to alum-bound allergen. Moreover, the CBP-Phl p 5-induced antibodies cross-reacted with five allergens from other grass species and were able to inhibit binding of human IgE to the allergen (Gronlund et al., 2002). To assess the influence of CBP on the nature of the T cell responses, the *in vitro* effect of CBP-Fel d 1 on human monocyte-derived dendritic cells (MDDCs) from healthy individuals was studied. The MDDCs readily phagocytosed the CBP and up-regulated CD86 but did not induce maturation of these cells. The increased release of TNF-α and IL-8 induced by the CBP-Fel d 1 was similar to that induced by naked Fel d 1. The authors suggested that CBP-couple allergen induced a semi-mature state of DCs, which could probably be associated with mixed Th1/Th2 responses or a regulatory response (Andersson et al., 2004).

Liposomes

Liposomes are small vesicles composed of naturally-derived phospholipids that are non-toxic and biodegradable. They can provide a depot effect after subcutaneous injection and act as potent immunological adjuvants, including the induction of Th1 immune responses. Liposomes have little allergenic potential (Arora and Gangal, 1991), and therefore, they have been proposed as carriers and immuno-adjuvants for allergens (Audera et al., 1991).

The modulation of allergen-specific responses to mite allergens encapsulated in liposomes has been studied both in mice and humans. The encapsulation of a recombinant precursor form of Der p 1, ProDer p 1, from the major mite allergen *Dermatophagoides pteronyssinus,* into cationic liposomes made of DiC14-amidine, prevented allergic responses to house dust mite allergens (Jacquet et al., 2005). The complexation of the allergen to the liposome was necessary to induce optimal responses. Naïve mice immunized with DiC14-amidine/allergen liposomes induced specific IgG2a antibodies but no IgE antibodies, moreover, IL-5 levels were reduced and IFN-γ levels increased. Similar results were obtained by encapsulating the *D. siboney* allergen into liposomes made of dipalmitoyl phosphatidylcholine (Calderon et al., 2006). Mice immunized with the encapsulated allergen had increased serum IgG2a/IgG1 ratio and reduced cellular infiltration in the lungs when compared to aluminum-adsorbed allergen.

In humans a double-blind, placebo-controlled trial using liposomes with encapsulated extract of *D. pteronyssinus* in asthmatic patients, showed less allergic symptoms and lower medication scores in the allergen-liposome group than in the control group receiving empty liposomes (Basomba et al., 2002). Patients receiving liposomal allergen also showed reduced immediate skin sensitivity, reduced late skin responses, as well as decreased bronchial sensitivity. Serum levels of specific IgG, especially IgG4, increased throughout the treatment, whereas IgE levels only increased transiently at the beginning of the treatment and then declined to baseline levels. Although the treatment was well tolerated with a low rate of systemic reactions and the build-up phase was reduced from the 13 weeks of conventional SIT to 8 weeks, no follow-ups were reported and no similar studies have since been published.

Virus-like particles (VLPs)

Some viral proteins, e.g. the hepatitis B core antigen and the bacteriophage capsid protein Qβ, spontaneously assemble into highly repetitive structures named virus-like

particles (VLPs). The presentation of an antigen in a highly ordered and repetitive manner induces strong antibody responses in mice, whereas the same antigen presented as a monomer is not immunogenic (Bachmann et al., 1993; Jegerlehner et al., 2002). Many fungi contain mycoviruses that have a protein p1 from the transposon Ty1, which can also assemble into VLPs. In fact, VLPs derived from this p1 protein have been used to carry allergenic peptides from Der p 1 (Hirschberg et al., 1999) and Asp f 2 from *Aspergillus fumigatus* (Svirshchevskaya et al., 2002). The immunization of previously Der p 1-sensitized mice with VLP carrying the immunodominant epitope of Der p 1 inhibited IL-5 production and reduced allergen-specific T cell proliferation. This suppression was CD4 mediated and no increase in IFN-γ levels was observed (Hirschberg et al., 1999). After a single injection of VLPs expressing a dominant T cell epitope of Asp f 2, decreased, although only transient, antigen-specific T and B cell responses were observed (Svirshchevskaya et al., 2002). More recently, the first human testing of Der p 1 covalently coupled to VLPs derived from the bacteriophage Qβ revealed good safety and strong immunogenicity (Kundig et al., 2006).

Antibodies

The humanized monoclonal anti-IgE antibody, omalizumab, was developed for the treatment of allergic diseases. Omalizumab binds to the Cε3 region of free IgE and thereby reduces its level in serum and down-regulates IgE receptors. Omalizumab reduced eosinophils, mast cells, T and B cells in nasal and bronchial tissues (Holgate et al., 2005). Moreover, the use of omalizumab as an add-on therapy to increase efficacy of other treatments has been described in several studies. A meta-analysis of seven studies conducted in patients with severe persistent asthma indicated that the addition of omalizumab to asthma treatment significantly reduced the asthma exacerbation rate and the incidence of emergency visits (Bousquet et al., 2005). A number of studies have assessed the potential of omalizumab in combination with SIT for allergic rhinitis. A recent double-blind, placebo-controlled trial using concomitantly omalizumab and rush immunotherapy in ragweed allergic patients showed that patients receiving the combined treatment had reduced seasonal allergic rhinitis severity scores and fewer adverse events than patients with only SIT (Casale et al., 2006). Further analysis of sera from those patients showed that IgG4 was only increased in SIT treated patients. Moreover, when SIT was used alone, only

partial inhibition of allergen-IgE binding was observed while treatment with omalizumab completely inhibited allergen-IgE binding (Klunker et al., 2007). This inhibition prevents binding of allergen-IgE complexes to the IgE receptors on mast cells and basophils reducing the release of mediators that cause the allergic reaction. An additional mechanism of action suggested for the anti-IgE treatment is the inhibition of IgE-mediated activation of allergen-specific T cell by antigen presenting cells, which express low and high affinity IgE receptors (van Neerven et al., 2006).

An anti-idiotype chimeric human IgG1 directed against IgE, 2G10, has also been shown to inhibit basophil activation (Wigginton et al., 2008). In contrast to omalizumab, the 2G10 antibody exert its effect by binding to surface bound IgE antibodies and cross-linking with the inhibitory receptor FcγRIIb receptor, which then inhibits degranulation of sensitized mast cells and basophils.

Other antibodies have been tested for their adjuvant or immunomodulatory effects, especially in therapeutic vaccination against chronic diseases or for the treatment of cancer or autoimmune diseases (Brekke and Sandlie, 2003). Such antibodies are typically directed towards cytokine receptors. Due to their key role in the pathophysiology of allergy and asthma, the cytokines derived from Th2 lymphocytes such as IL-4, IL-5, IL-9, IL-13 and IL-25 are especially interesting for SIT. IL-4 plays a critical role in the synthesis of IgE by B-lymphocytes, as well as its involvement in eosinophil recruitment to the airways. A unique function of IL-4 is to promote the differentiation of Th2 cells and therefore it acts at a proximal and critical site in the allergic response, making IL-4 an attractive target for inhibition. Indeed, IL-4 blocking antibodies inhibited allergen-induced airway hyper-responsiveness, goblet cell metaplasia and pulmonary eosinophilia in a murine model (Gavett et al., 1997).

Table 1. Strategies to improve SIT efficacy, safety and compliance.

SIT strategy	Method/Material	Advantages	Disadvantages
Route of administration	Subcutaneous	High success rate	Adverse events Long duration
	Sublingual SLIT; (Spit or Swallow)	Safety Convenience	Low efficacy Long duration High doses required
	Oral	Safety Convenience	Long duration High doses required
	Nasal	Targeting rhinitis?	Safety?
	Intranodal	Safety Short duration Compliance High efficacy Low dose	Ultrasound required
Allergen	Allergen extracts	Contains all allergens Mostly cheap	Ill-defined compositions
	Purified proteins	Well defined compositions	Purification expensive
	Recomb. Proteins	Structure flexibility Targeting sequences may be added	Costly production Only major allergens
	Synthetic peptides	Strong T-cell stimulation Safety	No B-cell stimulation Efficacy?
	DNA	Safety	Poor efficacy in humans Poor public acceptance of "Gene therapy"
Adjuvants	Mineral salts (Al^{3+} or Ca^{2+})	Depot General applicability	Poor Th1-triggereing
	TLR ligands	Strong Th1-triggering	No depot Toxicity
	Particles (Nano-/Micro-)	Draining to LN Depot Allergen stabilisation	Safety?
Antibodies	Anti-IgE	Safety Fast onset	Weak therapeutic effect Costly treatment
	Cytokines	Th1-triggering Strong immune modulation Potency	Unspecific immune modulation Toxicity

CONCLUSIONS

Subcutaneous allergen-specific immunotherapy is clearly established as an efficacious form of treatment for allergies, with the potential to modify the natural course of allergic rhinitis and possibly prevent the development of asthma in children. It is widely used around the world for a broad range of allergic conditions, including rhino-conjunctivitis, conjunctivitis, and asthma. For patients in whom pharmacotherapy fails to provide adequate symptom control, immunotherapy provides an effective alternative treatment option. Also, for patients with insect sting allergy, immunotherapy may be the only viable prophylactic treatment option.

The use of new technologies appears to offer the possibility for reducing the duration of the therapy, the number of injections required and, at the same time, improving safety. Research into alternative administration routes, e.g. sublingual and intranodal, is producing encouraging results. The convenience and safety profile are clearly superior to that of subcutaneous immunotherapy. If proved efficient, these new strategies should improve compliance, which is a critical issue in SIT. Especially children would benefit from substituting subcutaneous SIT with less invasive methods such as sublingual therapy. If immunomodulators and improved adjuvants can replace alum to increase efficacy with no loss of safety, this may eventually lead to injections becoming unnecessary and could even be used in a primary care setting rather than specialized allergy clinics. Immunotherapy may become suitable for more general, widespread use, particularly in children.

REFERENCES

Akdis, C.A. and Blaser, K. (2000) Mechanisms of allergen-specific immunotherapy. *Allergy*, **55**, 522-530.

Akdis, C.A., Joss, A., Akdis, M. and Blaser, K. (2001) Mechanism of IL-10-induced T cell inactivation in allergic inflammation and normal response to allergens. *Int Arch Allergy Immunol*, **124**, 180-182.

Akdis, C.A., Kussebi, F., Pulendran, B., Akdis, M., Lauener, R.P., Schmidt-Weber, C.B., Klunker, S., Isitmangil, G., Hansjee, N., Wynn, T.A., Dillon, S., Erb, P., Baschang, G., Blaser, K. and Alkan, S.S. (2003) Inhibition of T helper 2-type responses, IgE production and eosinophilia by synthetic lipopeptides. *Eur J Immunol*, **33**, 2717-2726.

Akira, S., Uematsu, S. and Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell*, **124**, 783-801.

Alexander, C., Kay, A.B. and Larche, M. (2002) Peptide-based vaccines in the treatment of specific allergy. *Curr Drug Targets Inflamm Allergy*, **1**, 353-361.

Andersson, T.N., Ekman, G.J., Gronlund, H., Buentke, E., Eriksson, T.L., Scheynius, A., Van Hage-Hamsten, M. and Gafvelin, G. (2004) A novel adjuvant-allergen complex, CBP-rFel d 1, induces up-regulation of CD86 expression and enhances cytokine release by human dendritic cells in vitro. *Immunology*, **113**, 253-259.

Andre, C., Perrin-Fayolle, M., Grosclaude, M., Couturier, P., Basset, D., Cornillon, J., Piperno, D., Girodet, B., Sanchez, R., Vallon, C., Bellier, P. and Nasr, M. (2003) A double-blind placebo-controlled evaluation of sublingual immunotherapy with a standardized ragweed extract in patients with seasonal rhinitis. Evidence for a dose-response relationship. *Int Arch Allergy Immunol*, **131**, 111-118.

Antje Heit, F.S.T.H.Dirk H.B.H.W. (2007) Antigen co-encapsulated with adjuvants efficiently drive protective T cell immunity. *European Journal of Immunology*, **37**, 2063-2074.

Arbos, P., Wirth, M., Arangoa, M.A., Gabor, F. and Irache, J.M. (2002) Gantrez(R) AN as a new polymer for the preparation of ligand-nanoparticle conjugates. *Journal of Controlled Release*, **83**, 321-330.

Arora, N. and Gangal, S.V. (1991) Liposomes as vehicle for allergen presentation in the immunotherapy of allergic diseases. *Allergy*, **46**, 386-392.

Audera, C., Ramirez, J., Soler, E. and Carreira, J. (1991) Liposomes as carriers for allergy immunotherapy. *Clin Exp Allergy*, **21**, 139-144.

Bachmann, M.F., Rohrer, U.H., Kundig, T.M., Burki, K., Hengartner, H. and Zinkernagel, R.M. (1993) The influence of antigen organization on B cell responsiveness. *Science*, **262**, 1448-1451.

Banerjee, B., Kelly, K.J., Fink, J.N., Henderson, J.D., Jr., Bansal, N.K. and Kurup, V.P. (2004) Modulation of airway inflammation by immunostimulatory CpG oligodeoxynucleotides in a murine model of allergic aspergillosis. *Infect Immun*, **72**, 6087-6094.

Barbey, C., Donatelli-Dufour, N., Batard, P., Corradin, G. and Spertini, F. (2004) Intranasal treatment with ovalbumin but not the major T cell epitope ovalbumin 323-339 generates interleukin-10 secreting T cells and results in the induction of allergen systemic tolerance. *Clin Exp Allergy*, **34**, 654-662.

Basomba, A., Tabar, A.I., de Rojas, D.H.F., Garcia, B.E., Alamar, R., Olaguibel, J.M., Prado, J.M.d., Martin, S. and Rico, P. (2002) Allergen vaccination with a liposome-encapsulated extract of Dermatophagoides pteronyssinus: A randomized, double-

blind, placebo-controlled trial in asthmatic patients. *Journal of Allergy and Clinical Immunology*, **109**, 943-948.

Batanero, E., Barral, P., Villalba, M. and Rodriguez, R. (2002) Biodegradable poly (DL-lactide glycolide) microparticles as a vehicle for allergen-specific vaccines: a study performed with Ole e 1, the main allergen of olive pollen. *J Immunol Methods*, **259**, 87-94.

Batanero, E., Barral, P., Villalba, M. and Rodriguez, R. (2003) Encapsulation of Ole e 1 in biodegradable microparticles induces Th1 response in mice: a potential vaccine for allergy. *J Control Release*, **92**, 395-398.

Bauchau, V. and Durham, S.R. (2004) Prevalence and rate of diagnosis of allergic rhinitis in Europe. *Eur Respir J*, **24**, 758-764.

Bauer, L., Bohle, B., Jahn-Schmid, B., Wiedermann, U., Daser, A., Renz, H., Kraft, D. and Ebner, C. (1997) Modulation of the allergic immune response in BALB/c mice by subcutaneous injection of high doses of the dominant T cell epitope from the major birch pollen allergen Bet v 1. *Clin Exp Immunol*, **107**, 536-541.

Bhalla, P.L., Swoboda, I. and Singh, M.B. (2001) Reduction in allergenicity of grass pollen by genetic engineering. *Int Arch Allergy Immunol*, **124**, 51-54.

Borchard, G. (2001) Chitosans for gene delivery. *Advanced Drug Delivery Reviews*, **52**, 145-150.

Bousquet, J., Cabrera, P., Berkman, N., Buhl, R., Holgate, S., Wenzel, S., Fox, H., Hedgecock, S., Blogg, M. and Cioppa, G.D. (2005) The effect of treatment with omalizumab, an anti-IgE antibody, on asthma exacerbations and emergency medical visits in patients with severe persistent asthma. *Allergy*, **60**, 302-308.

Bousquet, J., Hejjaoui, A., Soussana, M. and Michel, F.B. (1990) Double-blind, placebo-controlled immunotherapy with mixed grass-pollen allergoids. IV. Comparison of the safety and efficacy of two dosages of a high-molecular-weight allergoid. *J Allergy Clin Immunol*, **85**, 490-497.

Bousquet, J., Lockey, R. and Malling, H.J. (1998a) Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol*, **102**, 558-562.

Bousquet, J., Lockey, R., Malling, H.J., Alvarez-Cuesta, E., Canonica, G.W., Chapman, M.D., Creticos, P.J., Dayer, J.M., Durham, S.R., Demoly, P., Goldstein, R.J., Ishikawa, T., Ito, K., Kraft, D., Lambert, P.H., Lowenstein, H., Muller, U., Norman, P.S., Reisman, R.E., Valenta, R., Valovirta, E. and Yssel, H. (1998b) Allergen immunotherapy: therapeutic vaccines for allergic diseases. World Health Organization. American academy of Allergy, Asthma and Immunology. *Ann Allergy Asthma Immunol*, **81**, 401-405.

Bousquet, J., Lockey, R.F. and Malling, H.J. (1998c) WHO Position Paper. Allergen Immunotherapy: Therapeutic vaccines for allergic diseases. *Allergy*, **53**, 1-42.

Bousquet, J., Scheinmann, P., Guinnepain, M.T., Perrin-Fayolle, M., Sauvaget, J., Tonnel, A.B., Pauli, G., Caillaud, D., Dubost, R., Leynadier, F., Vervloet, D., Herman, D., Galvain, S. and Andre, C. (1999) Sublingual-swallow immunotherapy (SLIT) in patients with asthma due to house-dust mites: a double-blind, placebo-controlled study. *Allergy*, **54**, 249-260.

Brekke, O.H. and Sandlie, I. (2003) Therapeutic antibodies for human diseases at the dawn of the twenty-first century. *Nat Rev Drug Discov*, **2**, 52-62.

Briner, T.J., Kuo, M.C., Keating, K.M., Rogers, B.L. and Greenstein, J.L. (1993) Peripheral T-cell tolerance induced in naive and primed mice by subcutaneous injection of peptides from the major cat allergen Fel d I. *Proc Natl Acad Sci U S A*, **90**, 7608-7612.

Brugnolo, F., Sampognaro, S., Liotta, F., Cosmi, L., Annunziato, F., Manuelli, C., Campi, P., Maggi, E., Romagnani, S. and Parronchi, P. (2003) The novel synthetic immune response modifier R-848 (Resiquimod) shifts human allergen-specific CD4+ TH2 lymphocytes into IFN-gamma-producing cells. *J Allergy Clin Immunol*, **111**, 380-388.

Calderon, L., Facenda, E., Machado, L., Uyema, K., Rodriguez, D., Gomez, E., Martinez, Y., Gonzalez, B., Bourg, V., Alvarez, C., Otero, A., Russo, M., Labrada, A. and Lanio, M.E. (2006) Modulation of the specific allergic response by mite allergens encapsulated into liposomes. *Vaccine*, **24 Suppl 2**, S2-38-39.

Camporota, L., Corkhill, A., Long, H., Lordan, J., Stanciu, L., Tuckwell, N., Cross, A., Stanford, J.L., Rook, G.A., Holgate, S.T. and Djukanovic, R. (2003) The effects of Mycobacterium vaccae on allergen-induced airway responses in atopic asthma. *Eur Respir J*, **21**, 287-293.

Casale, T.B. (2004) Status of immunotherapy: current and future. *J Allergy Clin Immunol*, **113**, 1036-1039.

Casale, T.B., Busse, W.W., Kline, J.N., Ballas, Z.K., Moss, M.H., Townley, R.G., Mokhtarani, M., Seyfert-Margolis, V., Asare, A., Bateman, K. and Deniz, Y. (2006) Omalizumab pretreatment decreases acute reactions after rush immunotherapy for ragweed-induced seasonal allergic rhinitis. *J Allergy Clin Immunol*, **117**, 134-140.

Cezmi A. Akdis, F.K.B.P.M.A.Roger P.L.Carsten B.S.-W.S.K.G.I.N.H.Thomas A.W.S.D.P.E.G.B. (2003) Inhibition of T helper 2-type responses, IgE production and eosinophilia by synthetic lipopeptides. *European Journal of Immunology*, **33**, 2717-2726.

Chapman, M.D., Smith, A.M., Vailes, L.D., Arruda, L.K., Dhanaraj, V. and Pomes, A. (2000) Recombinant allergens for diagnosis and therapy of allergic disease. *J Allergy Clin Immunol*, **106**, 409-418.

Chew, J.L., Wolfowicz, C.B., Mao, H.-Q., Leong, K.W. and Chua, K.Y. (2003) Chitosan nanoparticles containing plasmid DNA encoding house dust mite allergen, Der p 1 for oral vaccination in mice. *Vaccine*, **21**, 2720-2729.

Chisholm, D., Libet, L., Hayashi, T. and Horner, A.A. (2004) Airway peptidoglycan and immunostimulatory DNA exposures have divergent effects on the development of airway allergen hypersensitivities. *J Allergy Clin Immunol*, **113**, 448-454.

Crameri, R., Fluckiger, S., Daigle, I., Kundig, T. and Rhyner, C. (2007) Design, engineering and in vitro evaluation of MHC class-II targeting allergy vaccines. *Allergy*, **62**, 197-206.

Creticos, P.S., Schroeder, J.T., Hamilton, R.G., Balcer-Whaley, S.L., Khattignavong, A.P., Lindblad, R., Li, H., Coffman, R., Seyfert, V., Eiden, J.J., Broide, D. and the Immune Tolerance Network, G. (2006) Immunotherapy with a Ragweed-Toll-Like Receptor 9 Agonist Vaccine for Allergic Rhinitis. *N Engl J Med*, **355**, 1445-1455.

de Weerd, N., Bhalla, P.L. and Singh, M.B. (2003) Oral immunization with a recombinant major grass pollen allergen induces blocking antibodies in mice. *Int Arch Allergy Immunol*, **130**, 119-124.

Degen, W.G., Jansen, T. and Schijns, V.E. (2003) Vaccine adjuvant technology: from mechanistic concepts to practical applications. *Expert Rev Vaccines*, **2**, 327-335.

Devos, R., Plaetinck, G., Cornelis, S., Guisez, Y., Van der Heyden, J. and Tavernier, J. (1995) Interleukin-5 and its receptor: a drug target for eosinophilia associated with chronic allergic disease. *J Leukoc Biol*, **57**, 813-819.

Di Rienzo, V., Marcucci, F., Puccinelli, P., Parmiani, S., Frati, F., Sensi, L., Canonica, G.W. and Passalacqua, G. (2003) Long-lasting effect of sublingual immunotherapy

in children with asthma due to house dust mite: a 10-year prospective study. *Clin Exp Allergy*, **33**, 206-210.

Drachenberg, K.J., Wheeler, A.W., Stuebner, P. and Horak, F. (2001) A well-tolerated grass pollen-specific allergy vaccine containing a novel adjuvant, monophosphoryl lipid A, reduces allergic symptoms after only four preseasonal injections. *Allergy*, **56**, 498-505.

Durham, S.R. and Till, S.J. (1998) Immunologic changes associated with allergen immunotherapy. *J Allergy Clin Immunol*, **102**, 157-164.

Durham, S.R., Varney, V.A., Gaga, M., Jacobson, M.R., Varga, E.M., Frew, A.J. and Kay, A.B. (1999a) Grass pollen immunotherapy decreases the number of mast cells in the skin. *Clin Exp Allergy*, **29**, 1490-1496.

Durham, S.R., Walker, S.M., Varga, E.M., Jacobson, M.R., O'Brien, F., Noble, W., Till, S.J., Hamid, Q.A. and Nouri-Aria, K.T. (1999b) Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med*, **341**, 468-475.

Eisenbarth, S.C., Piggott, D.A., Huleatt, J.W., Visintin, I., Herrick, C.A. and Bottomly, K. (2002) Lipopolysaccharide-enhanced, Toll-like Receptor 4-dependent T Helper Cell Type 2 Responses to Inhaled Antigen. *J. Exp. Med.*, **196**, 1645-1651.

Ewbank, P.A., Murray, J., Sanders, K., Curran-Everett, D., Dreskin, S. and Nelson, H.S. (2003) A double-blind, placebo-controlled immunotherapy dose-response study with standardized cat extract. *J Allergy Clin Immunol*, **111**, 155-161.

Fellrath, J.M., Kettner, A., Dufour, N., Frigerio, C., Schneeberger, D., Leimgruber, A., Corradin, G. and Spertini, F. (2003) Allergen-specific T-cell tolerance induction with allergen-derived long synthetic peptides: results of a phase I trial. *J Allergy Clin Immunol*, **111**, 854-861.

Ferreira, F., Ebner, C., Kramer, B., Casari, G., Briza, P., Kungl, A.J., Grimm, R., Jahn-Schmid, B., Breiteneder, H., Kraft, D., Breitenbach, M., Rheinberger, H.J. and Scheiner, O. (1998) Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *Faseb J*, **12**, 231-242.

Ferreira, F., Wallner, M., Breiteneder, H., Hartl, A., Thalhamer, J. and Ebner, C. (2002) Genetic engineering of allergens: future therapeutic products. *Int Arch Allergy Immunol*, **128**, 171-178.

Frankland, A.W. and Augustin, R. (1954) Prophylaxis of summer hay-fever and asthma: a controlled trial comparing crude grass-pollen extracts with the isolated main protein component. *Lancet*, **266**, 1055-1057.

Fratil, F., Incorvaia, C., Marcucci, F., Sensi, L., Di Cara, G., Puccinelli, P. and Dal Bo, S. (2006) Dose dependence of efficacy but not of safety in sublingual immunotherapy. *Monaldi Arch Chest Dis*, **65**, 38-40.

Freeman, J. (1911) Further observations on the treatment of hay fever by hypodermic inoculations of pollen vaccine. *Lancet* **178**, 814-817.

Freeman, J. (1914) Vaccination against hay fever. *Lancet* **183**, 1178-1180.

Gabor, F., Bogner, E., Weissenboeck, A. and Wirth, M. (2004) The lectin-cell interaction and its implications to intestinal lectin-mediated drug delivery. *Advanced Drug Delivery Reviews*, **56**, 459-480.

Gangloff, S.C. and Guenounou, M. (2004) Toll-like receptors and immune response in allergic disease. *Clin Rev Allergy Immunol*, **26**, 115-125.

Gavett, S.H., O'Hearn, D.J., Karp, C.L., Patel, E.A., Schofield, B.H., Finkelman, F.D. and Wills-Karp, M. (1997) Interleukin-4 receptor blockade prevents airway responses induced by antigen challenge in mice. *Am J Physiol*, **272**, L253-261.

Gidaro, G.B., Marcucci, F., Sensi, L., Incorvaia, C., Frati, F. and Ciprandi, G. (2005) The safety of sublingual-swallow immunotherapy: an analysis of published studies. *Clin Exp Allergy*, **35**, 565-571.

Gieni, R.S., Yang, X., Kelso, A. and Hayglass, K.T. (1996) Limiting dilution analysis of CD4 T-cell cytokine production in mice administered native versus polymerized ovalbumin: directed induction of T-helper type-1-like activation. *Immunology*, **87**, 119-126.

Golden, D.B. (2005) Insect sting allergy and venom immunotherapy: a model and a mystery. *J Allergy Clin Immunol*, **115**, 439-447; quiz 448.

Goldsby, R.A., Kindt, T.J., Osborne, B.A., Kuby, J. (2003) *Immunology*. W H Freeman & Co.

Gomez, S., Gamazo, C., Roman, B.S., Ferrer, M., Sanz, M.L. and Irache, J.M. (2007) Gantrez(R) AN nanoparticles as an adjuvant for oral immunotherapy with allergens. *Vaccine*, **25**, 5263-5271.

Grammer, L.C., Shaughnessy, M.A. and Patterson, R. (1987) Standardization of modified allergens using polymerized ragweed as a model system. *Clin Rev Allergy*, **5**, 107-116.

Gronlund, H., Vrtala, S., Wiedermann, U., Dekan, G., Kraft, D., Valenta, R. and Van Hage-Hamsten, M. (2002) Carbohydrate-based particles: a new adjuvant for allergen-specific immunotherapy. *Immunology*, **107**, 523-529.

Gupta, R.K. (1998) Aluminum compounds as vaccine adjuvants. *Adv Drug Deliv Rev*, **32**, 155-172.

Gupta, R.K., Relyveld, E.H., Lindblad, E.B., Bizzini, B., Ben-Efraim, S. and Gupta, C.K. (1993) Adjuvants--a balance between toxicity and adjuvanticity. *Vaccine*, **11**, 293-306.

Gutierro, I., Hernandez, R.M., Igartua, M., Gascon, A.R. and Pedraz, J.L. (2002) Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres. *Vaccine*, **21**, 67-77.

Haugaard, L., Dahl, R. and Jacobsen, L. (1993) A controlled dose-response study of immunotherapy with standardized, partially purified extract of house dust mite: clinical efficacy and side effects. *J Allergy Clin Immunol*, **91**, 709-722.

Heldwein, K.A. and Fenton, M.J. (2002) The role of Toll-like receptors in immunity against mycobacterial infection. *Microbes and Infection*, **4**, 937-944.

Hirschberg, S., Layton, G.T., Harris, S.J., Savage, N., Dallman, M.J. and Lamb, J.R. (1999) CD4+ T cells induced by virus-like particles expressing a major T cell epitope down-regulate IL-5 production in an ongoing immune response to Der p 1 independently of IFN-{gamma} production. *Int. Immunol.*, **11**, 1927-1934.

Hisbergues, M., Magi, M., Rigaux, P., Steuve, J., Garcia, L., Goudercourt, D., Pot, B., Pestel, J. and Jacquet, A. (2007) In vivo and in vitro immunomodulation of Der p 1 allergen-specific response by Lactobacillus plantarum bacteria. *Clinical & Experimental Allergy*, **37**, 1286-1295.

Holgate, S.T., Djukanovic, R., Casale, T. and Bousquet, J. (2005) Anti-immunoglobulin E treatment with omalizumab in allergic diseases: an update on anti-inflammatory activity and clinical efficacy. *Clin Exp Allergy*, **35**, 408-416.

Hoyne, G.F., O'Hehir, R.E., Wraith, D.C., Thomas, W.R. and Lamb, J.R. (1993) Inhibition of T cell and antibody responses to house dust mite allergen by inhalation of the dominant T cell epitope in naive and sensitized mice. *J Exp Med*, **178**, 1783-1788.

Hufnagl, K., Wagner, B., Winkler, B., Baier, K., Hochreiter, R., Thalhamer, J., Kraft, D., Scheiner, O., Breiteneder, H. and Wiedermann, U. (2003) Induction of mucosal

tolerance with recombinant Hev b 1 and recombinant Hev b 3 for prevention of latex allergy in BALB/c mice. *Clin Exp Immunol*, **133**, 170-176.

Hunter, S.K., Andracki, M.E. and Krieg, A.M. (2001) Biodegradable microspheres containing group B Streptococcus vaccine: Immune response in mice. *American Journal of Obstetrics and Gynecology*, **185**, 1174.

Illi, S., von Mutius, E., Lau, S., Bergmann, R., Niggemann, B., Sommerfeld, C. and Wahn, U. (2001) Early childhood infectious diseases and the development of asthma up to school age: a birth cohort study. *Bmj*, **322**, 390-395.

Incorvaia, C., Frati, F., Puccinelli, P., Riario-Sforza, G.G. and Dal Bo, S. (2006) Dose dependence of efficacy and safety of subcutaneous immunotherapy. *Monaldi Arch Chest Dis*, **65**, 41-43.

Iwasaki, A. and Medzhitov, R. (2004) Toll-like receptor control of the adaptive immune responses. *Nat Immunol*, **5**, 987-995.

Jackson, M. (2001) Allergy: the making of a modern plague. *Clin Exp Allergy*, **31**, 1665-1671.

Jacquet, A., Vanderschrick, J.-F., Vandenbranden, M., Elouahabi, A., Magi, M., Garcia, L. and Ruysschaert, J.-M. (2005) Vaccination with the recombinant allergen ProDer p 1 complexed with the cationic lipid DiC14-amidine prevents allergic responses to house dust mite. *Mol Ther*, **11**, 960-968.

Jahn-Schmid, B., Wiedermann, U., Bohle, B., Repa, A., Kraft, D. and Ebner, C. (1999) Oligodeoxynucleotides containing CpG motifs modulate the allergic TH2 response of BALB/c mice to Bet v 1, the major birch pollen allergen. *J Allergy Clin Immunol*, **104**, 1015-1023.

Jain, V.V., Kitagaki, K. and Kline, J.N. (2003) CpG DNA and immunotherapy of allergic airway diseases. *Clin Exp Allergy*, **33**, 1330-1335.

Janeway, C.H. (1989) Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol*, **54**, 1-13.

Janssen, E.M., Wauben, M.H., Jonker, E.H., Hofman, G., Van Eden, W., Nijkamp, F.P. and Van Oosterhout, A.J. (1999) Opposite effects of immunotherapy with ovalbumin and the immunodominant T-cell epitope on airway eosinophilia and hyperresponsiveness in a murine model of allergic asthma. *Am J Respir Cell Mol Biol*, **21**, 21-29.

Jayasekera, N.P., Toma, T.P., Williams, A. and Rajakulasingam, K. (2007) Mechanisms of immunotherapy in allergic rhinitis. *Biomedecine & Pharmacotherapy*, **61**, 29-33.

Jeannin, P., Lecoanet, S., Delneste, Y., Gauchat, J.F. and Bonnefoy, J.Y. (1998) IgE versus IgG4 production can be differentially regulated by IL-10. *J Immunol*, **160**, 3555-3561.

Jegerlehner, A., Maurer, P., Bessa, J., Hinton, H.J., Kopf, M. and Bachmann, M.F. (2007) TLR9 Signaling in B Cells Determines Class Switch Recombination to IgG2a. *J Immunol*, **178**, 2415-2420.

Jegerlehner, A., Tissot, A., Lechner, F., Sebbel, P., Erdmann, I., Kundig, T., Bachi, T., Storni, T., Jennings, G., Pumpens, P., Renner, W.A. and Bachmann, M.F. (2002) A molecular assembly system that renders antigens of choice highly repetitive for induction of protective B cell responses. *Vaccine*, **20**, 3104-3112.

Jilek, S., Barbey, C., Spertini, F. and Corthesy, B. (2001) Antigen-independent suppression of the allergic immune response to bee venom phospholipase A(2) by DNA vaccination in CBA/J mice. *J Immunol*, **166**, 3612-3621.

Jilek, S., Walter, E., Merkle, H.P. and Corthesy, B. (2004) Modulation of allergic responses in mice by using biodegradable poly(lactide-co-glycolide) microspheres. *J Allergy Clin Immunol*, **114**, 943-950.

Johansen, P., Gander, B., Merkle, H.P. and Sesardic, D. (2000a) Ambiguities in the preclinical quality assessment of microparticulate vaccines. *Trends Biotechnol*, **18**, 203-211.

Johansen, P., Haffner, A.C., Koch, F., Zepter, K., Erdmann, I., Maloy, K., Simard, J.J., Storni, T., Senti, G., Bot, A., Wuthrich, B. and Kundig, T.M. (2005a) Direct intralymphatic injection of peptide vaccines enhances immunogenicity. *Eur J Immunol*, **35**, 568-574.

Johansen, P., Men, Y., Merkle, H.P. and Gander, B. (2000b) Revisiting PLA/PLGA microspheres: an analysis of their potential in parenteral vaccination. *Eur J Pharm Biopharm*, **50**, 129-146.

Johansen, P., Senti, G., Martinez Gomez, J.M., Storni, T., von Beust, B.R., Wuthrich, B., Bot, A. and Kundig, T.M. (2005b) Toll-like receptor ligands as adjuvants in allergen-specific immunotherapy. *Clin Exp Allergy*, **35**, 1591-1598.

Johansen, P., Senti, G., Martinez Gomez, J.M., Wuthrich, B., Bot, A. and Kundig, T.M. (2005c) Heat denaturation, a simple method to improve the immunotherapeutic potential of allergens. *Eur J Immunol*, **35**, 3591-3598.

Joseph, A., Louria-Hayon, I., Plis-Finarov, A., Zeira, E., Zakay-Rones, Z., Raz, E., Hayashi, T., Takabayashi, K., Barenholz, Y. and Kedar, E. (2002) Liposomal immunostimulatory DNA sequence (ISS-ODN): an efficient parenteral and mucosal adjuvant for influenza and hepatitis B vaccines. *Vaccine*, **20**, 3342-3354.

Jutel, M., Jaeger, L., Suck, R., Meyer, H., Fiebig, H. and Cromwell, O. (2005) Allergen-specific immunotherapy with recombinant grass pollen allergens. *J Allergy Clin Immunol*, **116**, 608-613.

Kaisho, T. and Akira, S. (2006) Toll-like receptor function and signaling. *J Allergy Clin Immunol*, **117**, 979-987; quiz 988.

Kay, A.B. (2004) Allergen immunotherapy with cat allergen peptides. *Springer Semin Immunopathol*, **25**, 391-399.

Kazzaz, J., Singh, M., Ugozzoli, M., Chesko, J., Soenawan, E. and O'Hagan, D.T. (2006) Encapsulation of the immune potentiators MPL and RC529 in PLG microparticles enhances their potency. *Journal of Controlled Release*, **110**, 566-573.

Klunker, S., Saggar, L.R., Seyfert-Margolis, V., Asare, A.L., Casale, T.B., Durham, S.R. and Francis, J.N. (2007) Combination treatment with omalizumab and rush immunotherapy for ragweed-induced allergic rhinitis: Inhibition of IgE-facilitated allergen binding. *J Allergy Clin Immunol*, **120**, 688-695.

Koppelman, S.J., van Koningsveld, G.A., Knulst, A.C., Gruppen, H., Pigmans, I.G. and de Jongh, H.H. (2002) Effect of heat-induced aggregation on the IgE binding of patatin (Sol t 1) is dominated by other potato proteins. *J Agric Food Chem*, **50**, 1562-1568.

Krieg, A.M. (2006) Therapeutic potential of Toll-like receptor 9 activation. *Nat Rev Drug Discov*, **5**, 471-484.

Krieg, A.M., Yi, A.-K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A. and Klinman, D.M. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature*, **374**, 546.

Kumar, M., Kong, X., Behera, A.K., Hellermann, G.R., Lockey, R.F. and Mohapatra, S.S. (2003) Chitosan IFN-gamma-pDNA Nanoparticle (CIN) Therapy for Allergic Asthma. *Genet Vaccines Ther*, **1**, 3.

Kundig, T.M., Senti, G., Schnetzler, G., Wolf, C., Prinz Vavricka, B.M., Fulurija, A., Hennecke, F., Sladko, K., Jennings, G.T. and Bachmann, M.F. (2006) Der p 1 peptide on virus-like particles is safe and highly immunogenic in healthy adults. *J Allergy Clin Immunol*, **117**, 1470-1476.

Kussebi, F., Karamloo, F., Rhyner, C., Schmid-Grendelmeier, P., Salagianni, M., Mannhart, C., Akdis, M., Soldatova, L., Markovic-Housley, Z., von Beust, B.R., Kundig, T., Kemeny, D.M., Blaser, K., Crameri, R. and Akdis, C.A. (2005) A major allergen gene-fusion protein for potential usage in allergen-specific immunotherapy. *J Allergy Clin Immunol*, **115**, 323-329.

Larche, M., Akdis, C.A. and Valenta, R. (2006) Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol*, **6**, 761-771.

Lavelle, E.C. (2006) Lectins and microparticles for enhanced oral vaccination. *Methods*, **38**, 84-89.

Lemoine, D., Francois, C., Kedzierewicz, F., Preat, V., Hoffman, M. and Maincent, P. (1996) Stability study of nanoparticles of poly([var epsilon]-caprolactone), poly(d,l-lactide) and poly(d,l-lactide-co-glycolide). *Biomaterials*, **17**, 2191-2197.

Levings, M.K., Bacchetta, R., Schulz, U. and Roncarolo, M.G. (2002) The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells. *Int Arch Allergy Immunol*, **129**, 263-276.

Linhart, B., Hartl, A., Jahn-Schmid, B., Verdino, P., Keller, W., Krauth, M.T., Valent, P., Horak, F., Wiedermann, U., Thalhamer, J., Ebner, C., Kraft, D. and Valenta, R. (2005) A hybrid molecule resembling the epitope spectrum of grass pollen for allergy vaccination. *J Allergy Clin Immunol*, **115**, 1010-1016.

Litwin, A., Flanagan, M., Entis, G., Gottschlich, G., Esch, R., Gartside, P. and Michael, J.G. (1996) Immunologic effects of encapsulated short ragweed extract: a potent new agent for oral immunotherapy. *Ann Allergy Asthma Immunol*, **77**, 132-138.

Litwin, A., Flanagan, M., Entis, G., Gottschlich, G., Esch, R., Gartside, P. and Michael, J.G. (1997) Oral immunotherapy with short ragweed extract in a novel encapsulated preparation: a double-blind study. *J Allergy Clin Immunol*, **100**, 30-38.

Lombardi, C., Gargioni, S., Venturi, S., Zoccali, P., Canonica, G.W. and Passalacqua, G. (2001) Controlled study of preseasonal immunotherapy with grass pollen extract in tablets: effect on bronchial hyperreactivity. *J Investig Allergol Clin Immunol*, **11**, 41-45.

Magi, M., Garcia, L., Vandenbranden, M., Palmantier, R. and Jacquet, A. (2004) Heat denaturation affects the ProDer p 1 IgE reactivity and downregulates the development of the specific allergic response. *J Allergy Clin Immunol*, **114**, 545-552.

Malling, H.J. (2004) Comparison of the clinical efficacy and safety of subcutaneous and sublingual immunotherapy: methodological approaches and experimental results. *Curr Opin Allergy Clin Immunol*, **4**, 539-542.

Malling, H.J., Abreu-Nogueira, J., Alvarez-Cuesta, E., Bjorksten, B., Bousquet, J., Caillot, D., Canonica, G.W., Passalacqua, G., Saxonis-Papageorgiou, P. and Valovirta, E. (1998) Local immunotherapy. *Allergy*, **53**, 933-944.

Maloy, K.J., Erdmann, I., Basch, V., Sierro, S., Kramps, T.A., Zinkernagel, R.M., Oehen, S. and Kundig, T.M. (2001) Intralymphatic immunization enhances DNA vaccination. *Proc Natl Acad Sci U S A*, **98**, 3299-3303.

Mansouri, S., Lavigne, P., Corsi, K., Benderdour, M., Beaumont, E. and Fernandes, J.C. (2004) Chitosan-DNA nanoparticles as non-viral vectors in gene therapy: strategies to improve transfection efficacy. *European Journal of Pharmaceutics and Biopharmaceutics*, **57**, 1-8.

Marazuela, E.G., Prado, N., Moro, E., Fernandez-Garcia, H., Villalba, M., Rodriguez, R. and Batanero, E. (2008) Intranasal vaccination with poly(lactide-co-glycolide) microparticles containing a peptide T of Ole e 1 prevents mice against sensitization. *Clinical & Experimental Allergy*, **38**, 520-528.

Marciani, D.J. (2003) Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. *Drug Discov Today*, **8**, 934-943.

Martinez Gomez, J.M., Fischer, S., Csaba, N., Kundig, T.M., Merkle, H.P., Gander, B. and Johansen, P. (2007) A Protective Allergy Vaccine Based on CpG- and Protamine-Containing PLGA Microparticles. *Pharm Res*, **24**, 1927-1935.

Mascarell, L., Van Overtvelt, L., Lombardi, V., Razafindratsita, A., Moussu, H., Horiot, S., Chabre, H., Limal, D., Moutel, S., Bauer, J., Chiavaroli, C. and Moingeon, P. (2007) A synthetic triacylated pseudo-dipeptide molecule promotes Th1/TReg immune responses and enhances tolerance induction via the sublingual route. *Vaccine*, **26**, 108-118.

Matricardi, P.M., Rosmini, F., Panetta, V., Ferrigno, L. and Bonini, S. (2002) Hay fever and asthma in relation to markers of infection in the United States. *J Allergy Clin Immunol*, **110**, 381-387.

Matzinger, P. (2002) The danger model: a renewed sense of self. *Science*, **296**, 301-305.

Medicines, A.C.o.S.o. (1986) Desensitizing vaccines. *Br Med J*, **293**, 948.

Mothes, N., Heinzkill, M., Drachenberg, K.J., Sperr, W.R., Krauth, M.T., Majlesi, Y., Semper, H., Valent, P., Niederberger, V., Kraft, D. and Valenta, R. (2003) Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. *Clinical & Experimental Allergy*, **33**, 1198-1208.

Muller, U., Akdis, C.A., Fricker, M., Akdis, M., Blesken, T., Bettens, F. and Blaser, K. (1998) Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. *J Allergy Clin Immunol*, **101**, 747-754.

Nelson, H.S. (2005) Advances in upper airway diseases and allergen immunotherapy. *J Allergy Clin Immunol*, **115**, 676-684.

Niederberger, V., Horak, F., Vrtala, S., Spitzauer, S., Krauth, M.T., Valent, P., Reisinger, J., Pelzmann, M., Hayek, B., Kronqvist, M., Gafvelin, G., Gronlund, H., Purohit, A., Suck, R., Fiebig, H., Cromwell, O., Pauli, G., van Hage-Hamsten, M. and Valenta, R. (2004) Vaccination with genetically engineered allergens prevents progression of allergic disease. *Proc Natl Acad Sci U S A*, **101 Suppl 2**, 14677-14682.

Niederberger, V., Reisinger, J., Valent, P., Krauth, M.T., Pauli, G., van Hage, M., Cromwell, O., Horak, F. and Valenta, R. (2007) Vaccination with genetically modified birch pollen allergens: immune and clinical effects on oral allergy syndrome. *J Allergy Clin Immunol*, **119**, 1013-1016.

Noon, L. (1911) Prophylactic inoculation against hay fever. *Lancet*, **177**, 1572-1573.

Norman, P.S. (2004) Immunotherapy: 1999-2004. *J Allergy Clin Immunol*, **113**, 1013-1023; quiz 1024.

Norman, P.S., Ohman, J.L., Jr., Long, A.A., Creticos, P.S., Gefter, M.A., Shaked, Z., Wood, R.A., Eggleston, P.A., Hafner, K.B., Rao, P., Lichtenstein, L.M., Jones, N.H. and Nicodemus, C.F. (1996) Treatment of cat allergy with T-cell reactive peptides. *Am J Respir Crit Care Med*, **154**, 1623-1628.

Nouri-Aria, K.T., Wachholz, P.A., Francis, J.N., Jacobson, M.R., Walker, S.M., Wilcock, L.K., Staple, S.Q., Aalberse, R.C., Till, S.J. and Durham, S.R. (2004) Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol*, **172**, 3252-3259.

O'Hagan, D.T. (1998) Recent advances in vaccine adjuvants for systemic and mucosal administration. *J Pharm Pharmacol*, **50**, 1-10.

O'Hagan, D.T. and Valiante, N.M. (2003) Recent advances in the discovery and delivery of vaccine adjuvants. *Nat Rev Drug Discov*, **2**, 727-735.

Oshiba, A., Hamelmann, E., Bradley, K.L., Loader, J.E., Renz, H., Larsen, G.L. and Gelfand, E.W. (1996) Pretreatment with allergen prevents immediate hypersensitivity and airway hyperresponsiveness. *Am J Respir Crit Care Med*, **153**, 102-109.

Passalacqua, G., Guerra, L., Pasquali, M., Lombardi, C. and Canonica, G.W. (2004) Efficacy and safety of sublingual immunotherapy. *Ann Allergy Asthma Immunol*, **93**, 3-12; quiz 12-13, 103.

Patel, M., Xu, D., Kewin, P., Choo-Kang, B., McSharry, C., Thomson, N.C. and Liew, F.Y. (2005) TLR2 agonist ameliorates established allergic airway inflammation by promoting Th1 response and not via regulatory T cells. *J Immunol*, **174**, 7558-7563.

Patterson, R., Suszko, I.M., Pruzansky, J.J., Zeiss, C.R., Metzger, W.J. and Roberts, M. (1977) Polymerization of mixtures of grass allergens. *J Allergy Clin Immunol*, **59**, 314-319.

Peng, H.-J., Tsai, L.-C., Su, S.-N., Chang, Z.-N., Shen, H.-D., Chao, P.-L., Kuo, S.-W., Tsao, I.Y. and Hung, M.-W. (2004) Comparison of different adjuvants of protein and DNA vaccination for the prophylaxis of IgE antibody formation. *Vaccine*, **22**, 756-762.

Peyre, M., Audran, R., Estevez, F., Corradin, G., Gander, B., Sesardic, D. and Johansen, P. (2004) Childhood and malaria vaccines combined in biodegradable microspheres produce immunity with synergistic interactions. *J Control Release*, **99**, 345-355.

Peyre, M., Sesardic, D., Merkle, H.P., Gander, B. and Johansen, P. (2003) An experimental divalent vaccine based on biodegradable microspheres induces protective immunity against tetanus and diphtheria. *J Pharm Sci*, **92**, 957-966.

Ponsonby, A.L. and Kemp, A. (2008) Investigation of the hygiene hypothesis: current issues and future directions. *Allergy*, **63**, 506-508.

Purello-D'Ambrosio, F., Gangemi, S., Isola, S., La Motta, N., Puccinelli, P., Parmiani, S., Savi, E. and Ricciardi, L. (1999) Sublingual immunotherapy: a double-blind, placebo-controlled trial with Parietaria judaica extract standardized in mass units in patients with rhinoconjunctivitis, asthma, or both. *Allergy*, **54**, 968-973.

Quarcoo, D., Weixler, S., Joachim, R.A., Stock, P., Kallinich, T., Ahrens, B. and Hamelmann, E. (2004) Resiquimod, a new immune response modifier from the family of imidazoquinolinamines, inhibits allergen-induced Th2 responses, airway inflammation and airway hyper-reactivity in mice. *Clinical & Experimental Allergy*, **34**, 1314-1320.

Razafindratsita, A., Saint-Lu, N., Mascarell, L., Berjont, N., Bardon, T., Betbeder, D., Van Overtvelt, L. and Moingeon, P. (2007) Improvement of sublingual immunotherapy efficacy with a mucoadhesive allergen formulation. *J Allergy Clin Immunol*, **120**, 278-285.

Reese, G., Viebranz, J., Leong-Kee, S.M., Plante, M., Lauer, I., Randow, S., Moncin, M.S., Ayuso, R., Lehrer, S.B. and Vieths, S. (2005) Reduced allergenic potency of VR9-1, a mutant of the major shrimp allergen Pen a 1 (tropomyosin). *J Immunol*, **175**, 8354-8364.

Reisinger, J., Horak, F., Pauli, G., van Hage, M., Cromwell, O., Konig, F., Valenta, R. and Niederberger, V. (2005) Allergen-specific nasal IgG antibodies induced by vaccination with genetically modified allergens are associated with reduced nasal allergen sensitivity. *Journal of Allergy and Clinical Immunology*, **116**, 347-354.

Richardson, S.C., Kolbe, H.V. and Duncan, R. (1999) Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA. *Int J Pharm*, **178**, 231-243.

Robinson, D.S., Larche, M. and Durham, S.R. (2004) Tregs and allergic disease. *J Clin Invest*, **114**, 1389-1397.

Rolinck-Werninghaus, C., Hamelmann, E., Keil, T., Kulig, M., Koetz, K., Gerstner, B., Kuehr, J., Zielen, S., Schauer, U., Kamin, W., Von Berg, A., Hammermann, J., Weinkauf, B., Weidinger, G., Stenglein, S. and Wahn, U. (2004) The co-seasonal application of anti-IgE after preseasonal specific immunotherapy decreases ocular and nasal symptom scores and rescue medication use in grass pollen allergic children. *Allergy*, **59**, 973-979.

Roman, B.S., Espuelas, S., Gomez, S., Gamazo, C., Sanz, M.L., Ferrer, M. and Irache, J.M. (2007) Intradermal immunization with ovalbumin-loaded poly- -caprolactone microparticles conferred protection in ovalbumin-sensitized allergic mice. *Clinical & Experimental Allergy*, **37**, 287-295.

Roth-Walter, F., Scholl, I., Untersmayr, E., Ellinger, A., Boltz-Nitulescu, G., Scheiner, O., Gabor, F. and Jensen-Jarolim, E. (2005) Mucosal targeting of allergen-loaded microspheres by Aleuria aurantia lectin. *Vaccine*, **23**, 2703-2710.

Roy, K., Mao, H.Q., Huang, S.K. and Leong, K.W. (1999) Oral gene delivery with chitosan--DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat Med*, **5**, 387-391.

Sanchez-Monge, R., Blanco, C., Perales, A.D., Collada, C., Carrillo, T., Aragoncillo, C. and Salcedo, G. (2000) Class I chitinases, the panallergens responsible for the latex-fruit syndrome, are induced by ethylene treatment and inactivated by heating. *J Allergy Clin Immunol*, **106**, 190-195.

Santeliz, J.V., Nest, G.V., Traquina, P., Larsen, E. and Wills-Karp, M. (2002) Amb a 1-linked CpG oligodeoxynucleotides reverse established airway hyperresponsiveness in a murine model of asthma. *J Allergy Clin Immunol*, **109**, 455-462.

Schijns, V.E. (2000) Immunological concepts of vaccine adjuvant activity. *Curr Opin Immunol*, **12**, 456-463.

Schmid-Grendelmeier, P., Holzmann, D., Himly, M., Weichel, M., Tresch, S., Ruckert, B., Menz, G., Ferreira, F., Blaser, K., Wuthrich, B. and Crameri, R. (2003) Native Art v 1 and recombinant Art v 1 are able to induce humoral and T cell-mediated in vitro and in vivo responses in mugwort allergy. *J Allergy Clin Immunol*, **111**, 1328-1336.

Schoenwetter, W.F., Dupclay, L., Jr., Appajosyula, S., Botteman, M.F. and Pashos, C.L. (2004) Economic impact and quality-of-life burden of allergic rhinitis. *Curr Med Res Opin*, **20**, 305-317.

Scholl, I., Weissenbock, A., Forster-Waldl, E., Untersmayr, E., Walter, F., Willheim, M., Boltz-Nitulescu, G., Scheiner, O., Gabor, F. and Jensen-Jarolim, E. (2004) Allergen-loaded biodegradable poly(D,L-lactic-co-glycolic) acid nanoparticles down-regulate an ongoing Th2 response in the BALB/c mouse model. *Clin Exp Allergy*, **34**, 315-321.

Sel, S., Wegmann, M., Sel, S., Bauer, S., Garn, H., Alber, G. and Renz, H. (2007) Immunomodulatory Effects of Viral TLR Ligands on Experimental Asthma Depend on the Additive Effects of IL-12 and IL-10. *J Immunol*, **178**, 7805-7813.

Shibata, Y., Foster, L.A., Bradfield, J.F. and Myrvik, Q.N. (2000) Oral Administration of Chitin Down-Regulates Serum IgE Levels and Lung Eosinophilia in the Allergic Mouse. *J Immunol*, **164**, 1314-1321.

Shibata, Y., Metzger, W.J. and Myrvik, Q.N. (1997) Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production. *J Immunol*, **159**, 2462-2467.

Simons, F.E., Shikishima, Y., Van Nest, G., Eiden, J.J. and HayGlass, K.T. (2004) Selective immune redirection in humans with ragweed allergy by injecting Amb a 1 linked to immunostimulatory DNA. *J Allergy Clin Immunol*, **113**, 1144-1151.

Singla, A.K. and Chawla, M. (2001) Chitosan: some pharmaceutical and biological aspects--an update. *J Pharm Pharmacol*, **53**, 1047-1067.

Sledge, R. (1938) Treatment of hay-fever with alum-precipitated pollen. *US Naval Med Bull* **36**, 18.

Sokol, C.L., Barton, G.M., Farr, A.G. and Medzhitov, R. (2008) A mechanism for the initiation of allergen-induced T helper type 2 responses. *Nat Immunol*, **9**, 310-318.

Steinke, J.W., Rich, S.S. and Borish, L. (2008) 5. Genetics of allergic disease. *Journal of Allergy and Clinical Immunology*, **121**, S384-S387.

Storni, T., Kundig, T.M., Senti, G. and Johansen, P. (2005) Immunity in response to particulate antigen-delivery systems. *Adv Drug Deliv Rev*, **57**, 333-355.

Storni, T., Ruedl, C., Schwarz, K., Schwendener, R.A., Renner, W.A. and Bachmann, M.F. (2004) Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects. *J Immunol*, **172**, 1777-1785.

Strong, P., Clark, H. and Reid, K. (2002) Intranasal application of chitin microparticles down-regulates symptoms of allergic hypersensitivity to Dermatophagoides pteronyssinus and Aspergillus fumigatus in murine models of allergy. *Clin Exp Allergy*, **32**, 1794-1800.

Suzuki, M., Ohta, N., Min, W.-P., Matsumoto, T., Min, R., Zhang, X., Toida, K. and Murakami, S. (2007) Immunotherapy with CpG DNA conjugated with T-cell epitope peptide of an allergenic Cry j 2 protein is useful for control of allergic conditions in mice. *International Immunopharmacology*, **7**, 46-54.

Svirshchevskaya, E.V., Alekseeva, L., Marchenko, A., Viskova, N., Andronova, T.M., Benevolenskii, S.V. and Kurup, V.P. (2002) Immune response modulation by recombinant peptides expressed in virus-like particles. *Clinical & Experimental Immunology*, **127**, 199-205.

Swoboda, I., De Weerd, N., Bhalla, P.L., Niederberger, V., Sperr, W.R., Valent, P., Kahlert, H., Fiebig, H., Verdino, P., Keller, W., Ebner, C., Spitzauer, S., Valenta, R. and Singh, M.B. (2002) Mutants of the major ryegrass pollen allergen, Lol p 5, with reduced IgE-binding capacity: candidates for grass pollen-specific immunotherapy. *Eur J Immunol*, **32**, 270-280.

Swoboda, I., Grote, M., Verdino, P., Keller, W., Singh, M.B., De Weerd, N., Sperr, W.R., Valent, P., Balic, N., Reichelt, R., Suck, R., Fiebig, H., Valenta, R. and Spitzauer, S. (2004) Molecular characterization of polygalacturonases as grass pollen-specific marker allergens: expulsion from pollen via submicronic respirable particles. *J Immunol*, **172**, 6490-6500.

TePas, E.C., Hoyte, E.G., McIntire, J.J. and Umetsu, D.T. (2004) Clinical efficacy of microencapsulated timothy grass pollen extract in grass-allergic individuals. *Ann Allergy Asthma Immunol*, **92**, 25-31.

Terada, T., Zhang, K., Belperio, J., Londhe, V. and Saxon, A. (2006) A chimeric human-cat Fcγ-Fel d1 fusion protein inhibits systemic, pulmonary, and cutaneous allergic reactivity to intratracheal challenge in mice sensitized to Fel d1, the major cat allergen. *Clinical Immunology*, **120**, 45-56.

Thomson, N.C. (2007) The role of environmental tobacco smoke in the origins and progression of asthma. *Curr Allergy Asthma Rep*, **7**, 303-309.

Till, S.J., Francis, J.N., Nouri-Aria, K. and Durham, S.R. (2004) Mechanisms of immunotherapy. *J Allergy Clin Immunol*, **113**, 1025-1034; quiz 1035.

Tresch, S., Holzmann, D., Baumann, S., Blaser, K., Wuthrich, B., Crameri, R. and Schmid-Grendelmeier, P. (2003) In vitro and in vivo allergenicity of recombinant Bet v 1 compared to the reactivity of natural birch pollen extract. *Clin Exp Allergy*, **33**, 1153-1158.

Tulic, M.K., Fiset, P.O., Christodoulopoulos, P., Vaillancourt, P., Desrosiers, M., Lavigne, F., Eiden, J. and Hamid, Q. (2004) Amb a 1-immunostimulatory oligodeoxynucleotide conjugate immunotherapy decreases the nasal inflammatory response. *J Allergy Clin Immunol*, **113**, 235-241.

Tulic, M.K., Wale, J.L., Holt, P.G. and Sly, P.D. (2000) Modification of the Inflammatory Response to Allergen Challenge after Exposure to Bacterial Lipopolysaccharide. *Am. J. Respir. Cell Mol. Biol.*, **22**, 604-612.

Vajdy, M. and O'Hagan, D.T. (2001) Microparticles for intranasal immunization. *Adv Drug Deliv Rev*, **51**, 127-141.

Valenta, R. and Kraft, D. (2001) Recombinant allergen molecules: tools to study effector cell activation. *Immunol Rev*, **179**, 119-127.

Valenta, R. and Kraft, D. (2002) From allergen structure to new forms of allergen-specific immunotherapy. *Curr Opin Immunol*, **14**, 718-727.

Valenta, R. and Kraft, D. (2004) Recombinant allergens: from production and characterization to diagnosis, treatment, and prevention of allergy. *Methods*, **32**, 207-208.

Valenta, R. and Niederberger, V. (2007) Recombinant allergens for immunotherapy. *Journal of Allergy and Clinical Immunology*, **119**, 826-830.

Van Deusen, M.A., Angelini, B.L., Cordoro, K.M., Seiler, B.A., Wood, L. and Skoner, D.P. (1997) Efficacy and safety of oral immunotherapy with short ragweed extract. *Ann Allergy Asthma Immunol*, **78**, 573-580.

van Neerven, R.J., Knol, E.F., Ejrnaes, A. and Wurtzen, P.A. (2006) IgE-mediated allergen presentation and blocking antibodies: regulation of T-cell activation in allergy. *Int Arch Allergy Immunol*, **141**, 119-129.

van Neerven, R.J.J., Wikborg, T., Lund, G., Jacobsen, B., Brinch-Nielsen, A., Arnved, J. and Ipsen, H. (1999) Blocking Antibodies Induced by Specific Allergy Vaccination Prevent the Activation of CD4+ T Cells by Inhibiting Serum-IgE-Facilitated Allergen Presentation. *J Immunol*, **163**, 2944-2952.

Van Overtvelt, L., Lombardi, V., Razafindratsita, A., Saint-Lu, N., Horiot, S., Moussu, H., Mascarell, L. and Moingeon, P. (2008) IL-10-inducing adjuvants enhance sublingual immunotherapy efficacy in a murine asthma model. *Int Arch Allergy Immunol*, **145**, 152-162.

Vandenbulcke, L., Bachert, C., Van Cauwenberge, P. and Claeys, S. (2006) The innate immune system and its role in allergic disorders. *Int Arch Allergy Immunol*, **139**, 159-165.

Varde, N.K. and Pack, D.W. (2004) Microspheres for controlled release drug delivery. *Expert Opin Biol Ther*, **4**, 35-51.

Varney, V.A., Hamid, Q.A., Gaga, M., Ying, S., Jacobson, M., Frew, A.J., Kay, A.B. and Durham, S.R. (1993) Influence of grass pollen immunotherapy on cellular infiltration and cytokine mRNA expression during allergen-induced late-phase cutaneous responses. *J Clin Invest*, **92**, 644-651.

Villinger, F. (2003) Cytokines as clinical adjuvants: how far are we? *Expert Rev Vaccines*, **2**, 317-326.

Von Garnier, C., Astori, M., Kettner, A., Dufour, N., Corradin, G. and Spertini, F. (2002) In vivo kinetics of the immunoglobulin E response to allergen: bystander effect of coimmunization and relationship with anaphylaxis. *Clin Exp Allergy*, **32**, 401-410.

von Garnier, C., Astori, M., Kettner, A., Dufour, N., Heusser, C., Corradin, G. and Spertini, F. (2000) Allergen-derived long peptide immunotherapy down-regulates specific IgE response and protects from anaphylaxis. *Eur J Immunol*, **30**, 1638-1645.

Walker, C., Sawicka, E. and Rook, G.A. (2003) Immunotherapy with mycobacteria. *Curr Opin Allergy Clin Immunol*, **3**, 481-486.

Walter, F., Scholl, I., Untersmayr, E., Ellinger, A., Boltz-Nitulescu, G., Scheiner, O., Gabor, F. and Jensen-Jarolim, E. (2004) Functionalisation of allergen-loaded microspheres with wheat germ agglutinin for targeting enterocytes. *Biochem Biophys Res Commun*, **315**, 281-287.

Westritschnig, K., Focke, M., Verdino, P., Goessler, W., Keller, W., Twardosz, A., Mari, A., Horak, F., Wiedermann, U., Hartl, A., Thalhamer, J., Sperr, W.R., Valent, P. and Valenta, R. (2004) Generation of an allergy vaccine by disruption of the three-dimensional structure of the cross-reactive calcium-binding allergen, Phl p 7. *J Immunol*, **172**, 5684-5692.

Wheeler, A.W., Marshall, J.S. and Ulrich, J.T. (2001) A Th1-inducing adjuvant, MPL, enhances antibody profiles in experimental animals suggesting it has the potential to improve the efficacy of allergy vaccines. *Int Arch Allergy Immunol*, **126**, 135-139.

Wheeler, A.W. and Woroniecki, S.R. (2004) Allergy vaccines--new approaches to an old concept. *Expert Opin Biol Ther*, **4**, 1473-1481.

Wigginton, S.J., Furtado, P.B., Armour, K.L., Clark, M.R., Robins, A., Emara, M., Ghaemmaghami, A.M., Sewell, H.F. and Shakib, F. (2008) An immunoglobulin E-reactive chimeric human immunoglobulin G1 anti-idiotype inhibits basophil degranulation through cross-linking of FcepsilonRI with FcgammaRIIb. *Clin Exp Allergy*, **38**, 313-319.

Wild, C., Wallner, M., Hufnagl, K., Fuchs, H., Hoffmann-Sommergruber, K., Breiteneder, H., Scheiner, O., Ferreira, F. and Wiedermann, U. (2007) A recombinant allergen chimer as novel mucosal vaccine candidate for prevention of multi-sensitivities. *Allergy*, **62**, 33-41.

Williams, L.K., Ownby, D.R., Maliarik, M.J. and Johnson, C.C. (2005) The role of endotoxin and its receptors in allergic disease. *Ann Allergy Asthma Immunol*, **94**, 323-332.

Wills-Karp, M. (2004) Interleukin-13 in asthma pathogenesis. *Immunological Reviews*, **202**, 175-190.

Wilson, D.R., Lima, M.T. and Durham, S.R. (2005) Sublingual immunotherapy for allergic rhinitis: systematic review and meta-analysis. *Allergy*, **60**, 4-12.

Winkler, B., Baier, K., Wagner, S., Repa, A., Eichler, H.G., Scheiner, O., Kraft, D. and Wiedermann, U. (2002) Mucosal tolerance as therapy of type I allergy: intranasal application of recombinant Bet v 1, the major birch pollen allergen, leads to the suppression of allergic immune responses and airway inflammation in sensitized mice. *Clinical & Experimental Allergy*, **32**, 30-36.

Wischke, C., Borchert, H.-H., Zimmermann, J., Siebenbrodt, I. and Lorenzen, D.R. (2006) Stable cationic microparticles for enhanced model antigen delivery to dendritic cells. *Journal of Controlled Release*, **114**, 359-368.

Wu, B., Toussaint, G., Vander Elst, L., Granier, C., Jacquemin, M.G. and Saint-Remy, J.M. (2000) Major T cell epitope-containing peptides can elicit strong antibody responses. *Eur J Immunol*, **30**, 291-299.

Yang, I.A., Fong, K.M., Holgate, S.T. and Holloway, J.W. (2006) The role of Toll-like receptors and related receptors of the innate immune system in asthma. *Curr Opin Allergy Clin Immunol*, **6**, 23-28.

Yazdanbakhsh, M., Kremsner, P.G. and van Ree, R. (2002) Allergy, Parasites, and the Hygiene Hypothesis. *Science*, **296**, 490-494.

Yoshitomi, T., Nakagami, Y., Hirahara, K., Taniguchi, Y., Sakaguchi, M. and Yamashita, M. (2007) Intraoral administration of a T-cell epitope peptide induces immunological tolerance in Cry j 2-sensitized mice. *J Pept Sci*, **13**, 499-503.

Zhu, D., Kepley, C.L., Zhang, K., Terada, T., Yamada, T. and Saxon, A. (2005) A chimeric human-cat fusion protein blocks cat-induced allergy. *Nat Med*, **11**, 446-449.

Zuany-Amorim, C., Manlius, C., Trifilieff, A., Brunet, L.R., Rook, G., Bowen, G., Pay, G. and Walker, C. (2002) Long-term protective and antigen-specific effect of heat-killed Mycobacterium vaccae in a murine model of allergic pulmonary inflammation. *J Immunol*, **169**, 1492-1499.

CHAPTER 2

Intralymphatic injections as a new prospective administration route for allergen immunotherapy

Intralymphatic injections as a new prospective administration route for allergen immunotherapy

Julia M. Martínez-Gómez[1], Pål Johansen[1], Iris Erdmann[1], Reto Crameri[2], and Thomas M. Kündig[1]

[1] Unit for Experimental Immunotherapy, Department of Dermatology, University Hospital of Zurich, Gloriastrasse 31, 8091 Zurich, Switzerland.

[2] Swiss Institute of Allergy and Asthma Research (SIAF), Obere Strasse 22, 7270 Davos, Switzerland

Submitted for publication

ABSTRACT

Allergen-specific immunotherapy (SIT) is the only curative treatment for allergic patients and is typically performed by subcutaneous allergen injections. Although the prevalence of allergic diseases has doubled over the last 20 years, the number of patients undergoing SIT remains low, mainly due to its long duration and the risk of adverse events associated with subcutaneous SIT. In order to improve SIT safety and its attractiveness among patients, alternative routes of administrations have been proposed, e.g. the sublingual and nasal. The present study evaluated intralymphatic allergen administration as a novel method of SIT. In mice, direct injection of adjuvanted allergens into inguinal lymph nodes strongly enhanced allergen immunogenicity as compared to subcutaneous injections. Moreover, only intralymphatic injections induced production of IgG2a antibodies and it resulted in increased cytokine secretion than did subcutaneous administrations. Finally, intralymphatic immunotherapy caused improved protection against anaphylaxis than subcutaneous SIT. One reason for the superiority of intralymphatic injections was that this directs the antigen more efficiently to secondary lymphatic organs than subcutaneous injections, which caused antigens to remain at the injection site or to drain to the liver. In conclusion, direct allergen administration into lymph nodes increase SIT efficacy and therefore, represent a promising alternative to conventional SIT both in terms of efficacy and patient compliance.

INTRODUCTION

Treatments with corticosteroids and antihistamines can palliate symptoms associated with type I allergies but can not cure the disease. The only long-lasting treatment is allergen-specific immunotherapy (SIT), which is typically performed by subcutaneous administration of allergens. Despite the high efficacy of SIT, e.g. in *Hymenoptera*-venom allergy, the efficacy reaches 80-95% (Golden, 2005), only approximately 5% of allergic patients choose SIT as a first-line therapy, mainly due to the long duration of the treatment (3-5 years), the high number of injections required (30-80), and the risk of adverse allergic reactions. Therefore, there is a recognised interest in improving the current methods of SIT.

Numerous studies have investigated new strategies to optimise the allergen molecules (Jutel et al., 2005; Larche, 2007; Riemer et al., 2004), the adjuvants used (Creticos et al., 2004; Johansen et al., 2005b; Santeliz et al., 2002), the dosage form (Jilek et al., 2004; Kundig et al., 2006; Martinez Gomez et al., 2007), as well as the route of allergen administration (Barbey et al., 2004; Bohle et al., 2007; Razafindratsita et al., 2007).

Vaccines are typically administrated by subcutaneous injections. However, immune responses are induced in secondary lymphoid organs, where professional antigen presenting cells (APCs) present antigenic epitopes to the residing lymphocytes. It has been demonstrated that antigen localisation regulates immune responses and that even large amounts of antigens can be ignored by the immune system if they remain outside the organized lymphoid tissues (Zinkernagel, 2000; Zinkernagel et al., 1997). Hence, direct administration of antigens to lymph nodes would be a more efficient route to deliver antigens to the cells from the immune system. This concept has been shown valid for the administration of plasmid DNA (Maloy et al., 2001) and peptides (Johansen et al., 2005a) for the stimulation of strong and protective T-cell responses. In the present study, we compared direct intralymphatic and subcutaneous administration of allergens in mice as a means for improving SIT efficacy.

METHODS

Immunisation protocols

CBA female mice (6-8 weeks old, from Harlan, Horst, The Netherlands) were immunised three times with two week intervals in between by injection with the bee venom major allergen phospholipase A2 (PLA2, from Sigma-Aldrich, Buchs, Switzerland) or a recombinant Fel d 1 cat major allergen. Fel d 1 was engineered on a pQE30 expression vector (Qiagen, Hilden, Germany) containing a N-terminal $[His]_6$-tag for protein purification, and were produced in *Escherichia coli* as described (Crameri, Allergy 2007). The allergens were mixed with aluminium hydroxide (Alhydrogel 3%, from Brenntag Biosector, Fredrikssund, Denmark) and saline one hour before injection into the inguinal lymph node (i.l.) (Johansen et al., 2005a), subcutaneously (s.c.) or intraperitoneally (i.p.). The doses of aluminium hydroxide were 90 and 450 µg, for i.l. and s.c. or i.p. injections, respectively, and the injection volumes were 10 and 50 µl. Serum was prepared from tail vein blood drawn at different time points and frozen at -20°C until analysed by ELISA.

Alternatively, the two routes of administration were tested in a therapeutic model in CBA mice that were first sensitised to cat fur allergens by six weekly intraperitoneal (i.p.) injections of cat fur allergen extract from Stallergènes (Fresnes, France). The allergen extract (1 µg/dose) was adsorbed to aluminium hydroxide (900 µg/dose) and injected in a volume of 100 µl. Subsequently, the mice were desensitised with 1 µg Fel d 1 by i.l. or s.c. injections as described above. For induction of anaphylaxis, sensitised mice were challenged three weeks after the last desensitisation with 30 µg of cat fur allergen extract in saline (50 µl i.p.), and the body temperature was measured with a calibrated digital thermometer before and 30 minutes after the challenge.

Antibody measurements

For antibody detection, 96-well microtitre plates (Nunc Maxisorb, Basel, Switzerland) were coated with 5 µg/ml of PLA2 (Sigma-Aldrich) or 1 µg/ml cat fur allergen extract (Stallergènes) in carbonate buffer and incubated overnight at 4°C. After blocking the plates with 2.5% non-fat dry milk in PBS-0.05% Tween-20 (PBSTM), serial dilutions of individual sera in PBSTM were added to the plates, and incubated for 2 h. Then plates were incubated with biotinylated goat anti-mouse IgG1 or IgG2a (BD Pharmingen, San Diego, CA, USA) in PBSTM, followed by incubation with streptavidin-conjugated horseradish peroxidase (BD Pharmingen). In the last step, plates were incubated with the enzyme

substrate 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (Sigma-Aldrich) in 1 M sodium dihydrogen phosphate. The absorption was read at 405 nm on a Model 550 Microplate reader (BioRad, Hercules, CA, USA). Unless otherwise specified, all incubations were done at room temperature and were intercepted with PBST washes.

For detection of PLA2-specific IgE antibodies, plates were coated with 2 μg/ml of anti-mouse IgE capture antibody (BD Pharmingen). As secondary reagent for binding to mouse serum, an in-house biotinylated PLA2 was used at 3 μg/ml.

Cytokine-secretion assay

CBA mice were immunised three times by i.l. or s.c. injection with 1 μg of Fel d 1, as described above. One week after the last injection, spleens were isolated and single-cell suspensions prepared. Erythrocytes were removed by lysis (red blood cell lysis buffer, Sigma-Aldrich), and triplicates of $8x10^5$ cells were cultured with 10 μg/ml LoTox Fel d 1 from Indoors Biotechnology (Warminster, United Kingdom) or left un-stimulated in 200 μl of IMDM supplemented with 5% FCS, sodium pyruvate, L-Glutamine, penicillin and streptomycin. The cytokines secreted in the supernatant were measured using the DuoSet ELISA from R&D Systems (Abingdon, United Kingdom). Supernatants were collected after 20 h for IL-2 determination and after 72 h for determination of IL-4, IL-10 and IFN-γ.

Biodistribution

To compare the relative biodistribution of i.l. and s.c. administered proteins, CBA mice were injected with 99mTc pertechnetate-labelled human immunoglobulin (TechneScan® HIG) from Mallinckrodt Medical B.V. (Petten, the Netherlands); the half life of 99mTc is approx. six hours. The radioactive protein was injected in the inguinal lymph node or subcutaneously in the inguinal region at 3 MBq per dose. Four animals each were euthanised 90 min, and 17 hours after injection. Lungs, spleen, liver and inguinal lymph nodes were dissected, and analysed directly in a Cobra II gamma counter (Packard BioScience, Dreieich, Germany). The absolute distribution to each organ was expressed as radioactive count per minutes (cpm).

Statistics

Differences between independent groups were tested by non-parametric statistical analysis. Data were presented as means ± standard error and compared using a two-sided

independent Mann-Whitney U test or Kruskal-Wallis one-way analysis of variance with Dunn's multiple comparison test. The significance level was set at 5%.

RESULTS

Intralymphatic immunisation induced strong humoral responses at lower allergen doses

To asses the influence of the route of administration in the immunogenicity of allergens, CBA mice were immunised with 0.1, 1 or 10 μg of the bee venom major allergen PLA2 by three fortnightly s.c. or i.l. injections. As illustrated in Figure 1, i.l. administration of 0.1 μg PLA2 was sufficient to induce of high PLA2-specific IgG2a titers. Comparable titers upon s.c. or i.p. injections were obtained by injection of 10 μg PLA2. In contrast, significant titers of IgE were only observed after i.p. injection of PLA2.

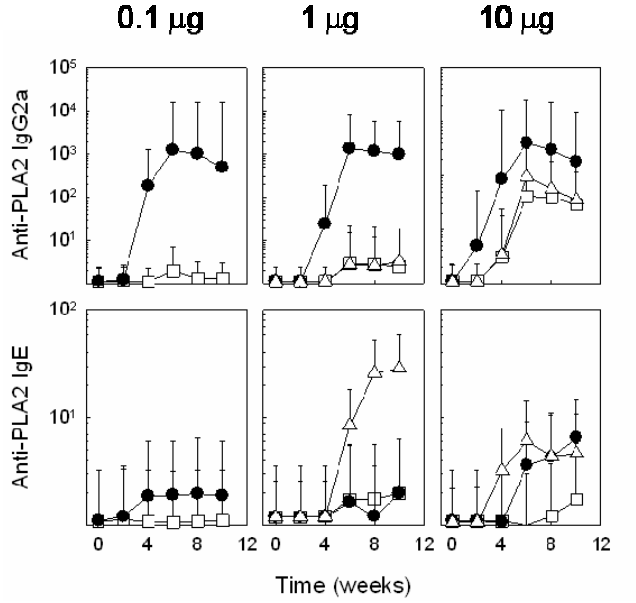

Figure 1. Bee venom PLA2-specific IgG2 and IgE antibodies measured by ELISA. Mice were immunized thrice with 0.1, 1, or 10 μg bee venom PLA2 adsorbed on aluminium hydroxide by intralymphatic (●), subcutaneous (□) or intraperitoneal (△) injections. Mice were bled at indicated time points and the sera analysed of PLA2-specific IgG2 and IgE. The data are illustrated as means ± SEM (n = 5).

The direct injection into inguinal lymph nodes was also tested using the perennial cat fur major allergen Fel d 1, which was injected in mice at two doses, 0.1 and 1 μg, and compared with the s.c. injection of 10 μg Fel d 1. As illustrated in Figure 2, comparable high IgG1 levels were induced in all three groups of mice, independent of the route of administration or the dose used. However, only mice that received Fel d 1 by i.l. injection produced IgG2a antibodies, and 0.1 μg was sufficient to stimulate a strong response. The response remained stable for at least eight weeks after the last injection (data not shown).

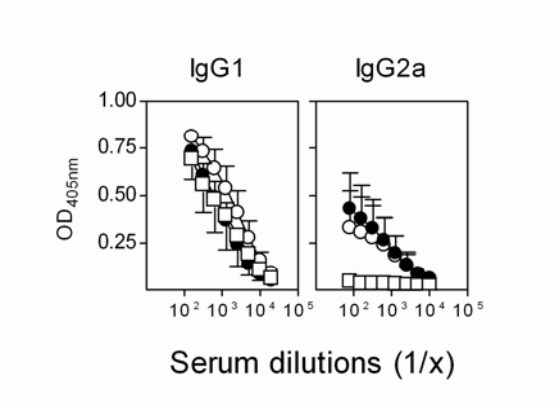

Figure 2. Cat allergen-specific IgG1 and IgG2a antibodies measured by ELISA. Mice were immunized thrice intralymphatically with 0.1 µg (●) or 1 µg (○) of Fel d 1 or subcutaneously (□) with 10 µg Fel d 1. The sera were analyzed after 2, 5 and 8 weeks of the last injection. The data from the last time point are illustrated here and expressed as means ± SEM (n = 4). One representative out of two independent experiments is shown.

Intralymphatic injections enhanced cytokine production

To evaluate the effect of the route of administration on the T-cell function, mice were immunised s.c. or i.l. with recombinant Fel d 1 protein. After seven days, splenocytes were re-stimulated in vitro with low endotoxin Fel d 1. The secretion of IL-2 was determined in the supernatants after 20 h, while IL-4, IL-10 and IFN-γ were measured after 72 h (Fig. 3). The secretion of all four cytokines was higher in cultured cells from mice that had been immunised by i.l. allergen administration.

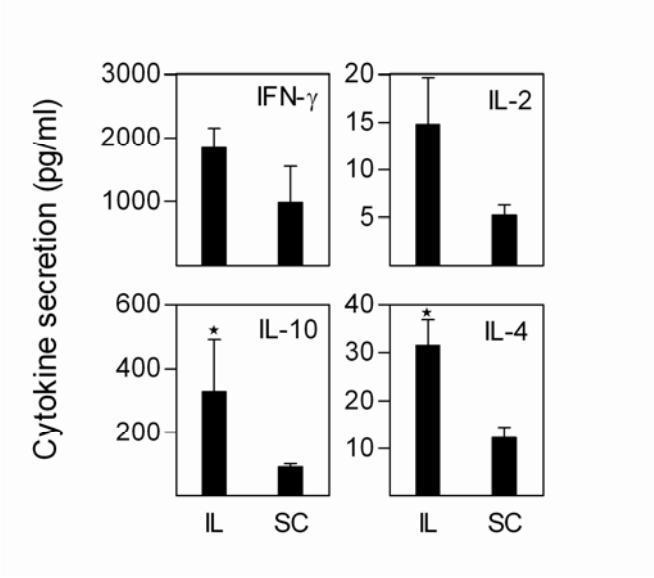

Figure 3. Cytokine secretion assay. Groups of four mice were immunised thrice with 1 µg of Fel d 1 intralymphatically or subcutaneously, and the splenocytes were analyzed for secretion of different cytokines after *in vitro* re-stimulation with 10 µg/ml of Fel d 1. The cytokine concentrations in supernatant are shown after subtraction of spontaneous secretion from non-stimulated splenocytes and as measured by ELISA. Statistical differences are indicated when $p<0.05$ as analysed by the Mann Whitney U test (n = 4).

Immunotherapy with intralymphatic Fel d 1 enhanced protection against anaphylaxis

Mice sensitised to cat fur allergen extract have high levels of serum IgE and IgG1, but no detectable IgG2a. Upon desensitisation with the Fel d 1 allergen, the i.l. route of administration induced a higher IgG2a response than did s.c. injections (data not shown). Three weeks after completed immunotherapy, mice were challenged with a high dose of cat fur allergen extract and subsequently monitored for anaphylaxis. As illustrated in Figure 4, immunotherapy performed by i.l. administration of the Fel d 1 caused significant improvement of the allergic status, these mice showed significantly less anaphylactic reaction than sensitised mice that did not received immunotherapy (p=0.048). In contrast, immunotherapy by means of three s.c. injections of the allergen was not sufficient to ameliorate anaphylactic reactions in sensitised mice.

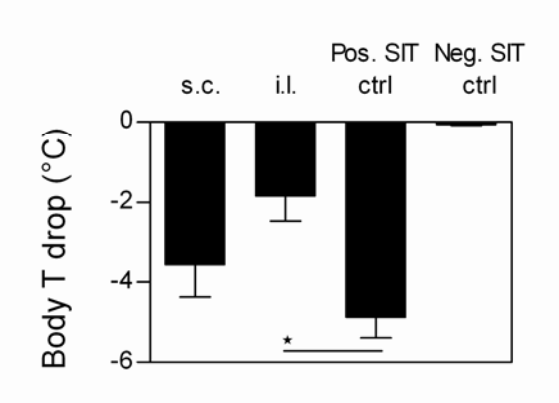

Figure 4. Allergen-specific immunotherapy and test of anaphylaxis. Groups of four mice were sensitised against Fel d 1 by i.p. injections of cat fur allergen extract, and then treated by i.l. or s.c. injections of recombinant Fel d 1. Four weeks after the last SIT injection, mice were challenge with 30 µg of cat fur allergen extract. The negative control group contained naive mice, while the positive control was a group of sensitised mice that did not receive immunotherapy. The body temperature drop was measured before and 30 minutes after the challenge, and the groups were compared by Kruskal-Wallis with a Dunn's post test of variance (* $p<0.05$).

Preferential distribution to spleen upon intralymphatic injection

One possible explanation to the observed benefit of i.l. immunisation and immunotherapy over s.c. injections could be that the latter administration route does not allow complete drainage of the allergen into secondary lymphatic tissues. This difference in allergen dose available for stimulation of allergen-specific lymphocytes may also lead to the preferential Th1 immune responses observed after i.l. allergen administration. Hence, to analyse the fate of an injected protein as a function of administration route, mice received i.l. or s.c. injections of a human immunoglobulin labelled with radioactive technetium. The analysis of the radioactivity in different tissues 90 min after the injection revealed that the

i.l. administration lead to a preferential accumulation of radioactivity in secondary lymphatic organs such as the inguinal lymph nodes and spleen ($p<0.05$). In contrast, s.c. administered protein more strongly drained to the liver ($p<0.05$). Seventeen hours after injection, significantly more radioactivity was recorded in the inguinal lymph node of i.l. than in the s.c. injected mice ($p<0.05$).

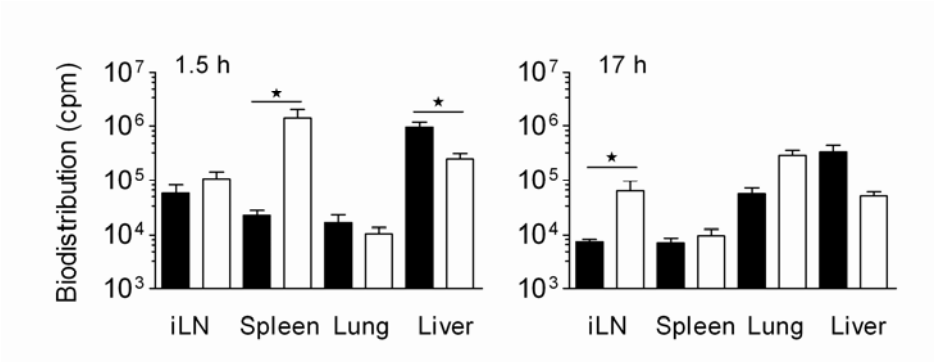

Figure 5. Biodistribution of intralymphatically and subcutaneously injected protein. Groups of four mice were injected with 99mTc-labelled human immunoglobulin directly into an inguinal lymph node (open bars) or subcutaneously in the inguinal region (filled bars). After 90 min and after 17 hours, the mice were euthanized, and the inguinal lymph nodes, the spleen, the lungs as well as all liver lobes were isolated and immediately assayed for gamma emmision. Statistical differences are indicated when $p<0.05$ as analysed by Mann Whitney U test ($n = 4$).

DISCUSSION

There is a high prevalence of allergic diseases in industrialised countries, with up to 20% of the population suffering from type I hypersensitivity reactions (Bauchau and Durham, 2004). The only curative treatment available for these patients is SIT (Durham et al., 1999). However, the benefits of SIT are arguably questionable with regard to the long-term commitment of the patient, the high treatment costs, and the evident risk of allergic adverse reactions associated with the immunotherapy (Casale, 2004). One important approach for improving SIT focuses on the route of administration of allergens. In this respect, sublingual immunotherapy (SLIT), in which the allergen is given as soluble tablets or drops, and local nasal immunotherapy have proven clinically effective (Bousquet et al., 1998; Bousquet et al., 1999; Nelson, 2005; Passalacqua et al., 2004). However, the treatment duration of SLIT and nasal SIT remains similar to that of current subcutaneous SIT (Canonica and Passalacqua, 2003) and the allergen dose is at least 50-100 times higher than the dose needed for subcutaneous immunisation (Bousquet et al., 2001).

The results presented in the current study showed that direct intralymphatic injections enhanced immunogenicity of allergens as compared to the classical SIT by the subcutaneous route of administration. These results are in line with already published data showing enhanced immunogenicity in both plasmid DNA (Maloy et al., 2001) and peptide (Johansen et al., 2005a) vaccination after direct intralymphatic injections. In addition, intranodal administration required at least 100 times less antigen (Maloy et al., 2001) or immune stimulatory molecules (von Beust et al., 2005) to induce potent cytotoxic T-cell immune responses than subcutaneous antigen administration. In the present study, intralymphatic immunisation was shown to stimulate humoral immune responses comparable to that of subcutaneous SIT, but with only 1% of the allergen dose. Moreover, while subcutaneous SIT only induced IgG1, the Th2-associated subclass, intralymphatic allergen administration also stimulated Th1-associated specific IgG2a antibodies. The enhanced humoral immune responses observed after intranodal immunisation were furthermore supported by a general increase in the secretion of cytokines after re-stimulation of the lymphocytes *in vitro*.

The geographical concept of immune reactivity emphasises the importance of antigen localisation (Zinkernagel, 2000). As conventional T- and B- cell responses can only be induced in organised lymphoid tissues, the direct injection of antigens into subcutaneous

lymph nodes would obviously facilitate the stimulation of stronger immune responses. Against early older assumptions, studies have also shown that antigen presentation does not have to take place in peripheral tissues, such as the skin, but that dendritic cells and other professional antigen-presenting cells residing in the lymph nodes and the spleen are capable of presenting antigen epitopes and to stimulate strong B- and T-cell responses (Heinzerling et al., 2006; Johansen et al., 2005a; Maloy et al., 2001; von Beust et al., 2005).

Hence, the reason why the intralymphatic route of immunisation was more efficient than subcutaneous allergen administration in stimulating strong immune responses, especially of the Th1-type, is most likely a reason of stochastic matter. As illustrated in the biodistribution of radioactive labelled proteins, a few percent of the subcutaneously injected material drained and remained in the lymphatic tissue (spleen and inguinal lymph nodes), while most of the material ended up in the liver. In contrast, after intralymphatic administration, the entire allergen dose is available for antigen-presentation in the lymph node. This, and the fact that high antigen doses favours Th1-like immune responses (Powell et al., 2007; Ruedl et al., 2000) explain why efficient lymphatic targeting of the allergen is important for the stimulation of protective immune responses in the allergen-specific immunotherapy. Indeed, such targeting has been applied by other means, for instance by using allergens bound to so-called virus like particles that very efficiently drain to the lymph nodes (Kundig et al., 2006; Vania Manolova, 2008). In the same way other kind of particles or carrier molecules can be imagined effective in strengthening immunotherapy solely by the fact that they bring more allergen into the secondary lymphatic tissue.

Another key point in immune reactivity is the duration of receptor stimulation in the immunological synapsis between lymphocytes and antigen-presenting cells. If the stimulation threshold is not reached, the lymphocytes will not be activated or only poorly activated (Giandomenica Iezzi, 1999; Huppa et al., 2003). Hence, as the intralymphatic allergen administration provides a larger effective allergen dose to the immunological synapsis, this increases the probability of obtaining sufficient synapsis time and lymphocyte stimulation/activation as compared to subcutaneous immunotherapy.

An additional important benefit of intralymphatic over subcutaneous immunotherapy is that the former avoids direct contact with the skin mast cells and therefore, prevents local adverse events associated with the allergen-mediated degranulation of such cells. Moreover, as lower therapeutic doses are required to induce comparably strong immune

responses, the intralymphatic immunotherapy may further reduce the risk of local and systemic allergic side effects to a minimum. Recently, the intralymphatic immunotherapy has been tested in humans for the treatment of both grass pollen and bee venom allergies (Senti et al., papers in preparation), demonstrating its clinical feasibility. The injection into subcutaneous lymph nodes has also been applied in the treatment of other conditions (Spaner et al., 2006); in all these human studies, the intralymphatic injections were guided and documented by ultrasound.

In closing, the intralymphatic route of administration represents a powerful tool to increase the efficacy of allergen-specific immunotherapy and to reduce the risk of adverse events as compared to conventional subcutaneous immunotherapy. In humans, this should potentially increase the attractiveness and compliance among patients otherwise reluctant to go through years and dozens of doctor visits and subcutaneous injections.

Acknowledgements

The authors thank Mrs. María J. Pena Rodríguez for her technical support, and Prof. Adriano Aguzzi for helpful discussions.

Author contributions

I performed all the experiments that involved the Fel d 1 protein and wrote the manuscript; Iris Erdmann performed the immunogenicity experiment with the PLA2; Pal Johansen performed the biodistribution experiment and wrote the manuscript; Reto Crameri prepared the recombinant Fel d 1 allergen; Thomas M. Kündig initiated and designed research and corrected the manuscript.

REFERENCES

Barbey, C., Donatelli-Dufour, N., Batard, P., Corradin, G. and Spertini, F. (2004) Intranasal treatment with ovalbumin but not the major T cell epitope ovalbumin 323-339 generates interleukin-10 secreting T cells and results in the induction of allergen systemic tolerance. *Clin Exp Allergy*, **34**, 654-662.

Bauchau, V. and Durham, S.R. (2004) Prevalence and rate of diagnosis of allergic rhinitis in Europe. *Eur Respir J*, **24**, 758-764.

Bohle, B., Kinaciyan, T., Gerstmayr, M., Radakovics, A., Jahn-Schmid, B. and Ebner, C. (2007) Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. *J Allergy Clin Immunol*, **120**, 707-713.

Bousquet, J., Lockey, R.F. and Malling, H.J. (1998) WHO Position Paper. Allergen Immunotherapy: Therapeutic vaccines for allergic diseases. *Allergy*, **53**, 1-42.

Bousquet, J., Scheinmann, P., Guinnepain, M.T., Perrin-Fayolle, M., Sauvaget, J., Tonnel, A.B., Pauli, G., Caillaud, D., Dubost, R., Leynadier, F., Vervloet, D., Herman, D., Galvain, S. and Andre, C. (1999) Sublingual-swallow immunotherapy (SLIT) in patients with asthma due to house-dust mites: a double-blind, placebo-controlled study. *Allergy*, **54**, 249-260.

Bousquet, J., Van Cauwenberge, P. and Khaltaev, N. (2001) Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol*, **108**, S147-334.

Canonica, G.W. and Passalacqua, G. (2003) Noninjection routes for immunotherapy. *Journal of Allergy and Clinical Immunology*, **111**, 437-448.

Casale, T.B. (2004) Status of immunotherapy: current and future. *J Allergy Clin Immunol*, **113**, 1036-1039.

Creticos, P.S., Chen, Y.H. and Schroeder, J.T. (2004) New approaches in immunotherapy: allergen vaccination with immunostimulatory DNA. *Immunol Allergy Clin North Am*, **24**, 569-581, v.

Durham, S.R., Walker, S.M., Varga, E.M., Jacobson, M.R., O'Brien, F., Noble, W., Till, S.J., Hamid, Q.A. and Nouri-Aria, K.T. (1999) Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med*, **341**, 468-475.

Giandomenica Iezzi, E.S.D.S.A.L. (1999) The interplay between the duration of TCR and cytokine signaling determines T cell polarization. *European Journal of Immunology*, **29**, 4092-4101.

Golden, D.B. (2005) Insect sting allergy and venom immunotherapy: a model and a mystery. *J Allergy Clin Immunol*, **115**, 439-447; quiz 448.

Heinzerling, L., Basch, V., Maloy, K., Johansen, P., Senti, G., Wuthrich, B., Storni, T. and Kundig, T.M. (2006) Critical role for DNA vaccination frequency in induction of antigen-specific cytotoxic responses. *Vaccine*, **24**, 1389-1394.

Huppa, J.B., Gleimer, M., Sumen, C. and Davis, M.M. (2003) Continuous T cell receptor signaling required for synapse maintenance and full effector potential. *Nat Immunol*, **4**, 749-755.

Jilek, S., Walter, E., Merkle, H.P. and Corthesy, B. (2004) Modulation of allergic responses in mice by using biodegradable poly(lactide-co-glycolide) microspheres. *J Allergy Clin Immunol*, **114**, 943-950.

Johansen, P., Haffner, A.C., Koch, F., Zepter, K., Erdmann, I., Maloy, K., Simard, J.J., Storni, T., Senti, G., Bot, A., Wuthrich, B. and Kundig, T.M. (2005a) Direct intralymphatic injection of peptide vaccines enhances immunogenicity. *Eur J Immunol*, **35**, 568-574.

Johansen, P., Senti, G., Martinez Gomez, J.M., Storni, T., von Beust, B.R., Wuthrich, B., Bot, A. and Kundig, T.M. (2005b) Toll-like receptor ligands as adjuvants in allergen-specific immunotherapy. *Clin Exp Allergy*, **35**, 1591-1598.

Jutel, M., Jaeger, L., Suck, R., Meyer, H., Fiebig, H. and Cromwell, O. (2005) Allergen-specific immunotherapy with recombinant grass pollen allergens. *J Allergy Clin Immunol*, **116**, 608-613.

Kundig, T.M., Senti, G., Schnetzler, G., Wolf, C., Prinz Vavricka, B.M., Fulurija, A., Hennecke, F., Sladko, K., Jennings, G.T. and Bachmann, M.F. (2006) Der p 1 peptide on virus-like particles is safe and highly immunogenic in healthy adults. *J Allergy Clin Immunol*, **117**, 1470-1476.

Larche, M. (2007) Update on the current status of peptide immunotherapy. *J Allergy Clin Immunol*, **119**, 906-909.

Maloy, K.J., Erdmann, I., Basch, V., Sierro, S., Kramps, T.A., Zinkernagel, R.M., Oehen, S. and Kundig, T.M. (2001) Intralymphatic immunization enhances DNA vaccination. *Proc Natl Acad Sci U S A*, **98**, 3299-3303.

Martinez Gomez, J.M., Fischer, S., Csaba, N., Kundig, T.M., Merkle, H.P., Gander, B. and Johansen, P. (2007) A Protective Allergy Vaccine Based on CpG- and Protamine-Containing PLGA Microparticles. *Pharm Res*, **24**, 1927-1935.

Nelson, H.S. (2005) Advances in upper airway diseases and allergen immunotherapy. *J Allergy Clin Immunol*, **115**, 676-684.

Passalacqua, G., Guerra, L., Pasquali, M., Lombardi, C. and Canonica, G.W. (2004) Efficacy and safety of sublingual immunotherapy. *Ann Allergy Asthma Immunol*, **93**, 3-12; quiz 12-13, 103.

Powell, R.J., Frew, A.J., Corrigan, C.J. and Durham, S.R. (2007) Effect of grass pollen immunotherapy with Alutard SQ on quality of life in seasonal allergic rhinoconjunctivitis. *Allergy*, **62**, 1335-1338.

Razafindratsita, A., Saint-Lu, N., Mascarell, L., Berjont, N., Bardon, T., Betbeder, D., Van Overtvelt, L. and Moingeon, P. (2007) Improvement of sublingual immunotherapy efficacy with a mucoadhesive allergen formulation. *J Allergy Clin Immunol*, **120**, 278-285.

Riemer, A., Scheiner, O. and Jensen-Jarolim, E. (2004) Allergen mimotopes. *Methods*, **32**, 321-327.

Ruedl, C., Bachmann, M.F. and Kopf, M. (2000) The antigen dose determines T helper subset development by regulation of CD40 ligand. *Eur J Immunol*, **30**, 2056-2064.

Santeliz, J.V., Nest, G.V., Traquina, P., Larsen, E. and Wills-Karp, M. (2002) Amb a 1-linked CpG oligodeoxynucleotides reverse established airway hyperresponsiveness in a murine model of asthma. *J Allergy Clin Immunol*, **109**, 455-462.

Spaner, D.E., Astsaturov, I., Vogel, T., Petrella, T., Elias, I., Burdett-Radoux, S., Verma, S., Iscoe, N., Hamilton, P. and Berinstein, N.L. (2006) Enhanced viral and tumor immunity with intranodal injection of canary pox viruses expressing the melanoma antigen, gp100. *Cancer*, **106**, 890-899.

Vania Manolova, A.F.M.B.K.S.P.S.Martin F.B. (2008) Nanoparticles target distinct dendritic cell populations according to their size. *European Journal of Immunology*, **38**, 1404-1413.

von Beust, B.R., Johansen, P., Smith, K.A., Bot, A., Storni, T. and Kundig, T.M. (2005) Improving the therapeutic index of CpG oligodeoxynucleotides by intralymphatic administration. *Eur J Immunol*, **35**, 1869-1876.

Zinkernagel, R.M. (2000) Localization dose and time of antigens determine immune reactivity. *Semin Immunol*, **12**, 163-171; discussion 257-344.

Zinkernagel, R.M., Ehl, S., Aichele, P., Oehen, S., Kundig, T. and Hengartner, H. (1997) Antigen localisation regulates immune responses in a dose- and time-dependent fashion: a geographical view of immune reactivity. *Immunol Rev*, **156**, 199-209.

CHAPTER 3

Targeting the MHC class II pathway of antigen presentation enhances immunogenicity and safety of allergen immunotherapy

Targeting the MHC class II pathway of antigen presentation enhances immunogenicity and safety of allergen immunotherapy

Julia M. Martínez-Gómez[1], Pål Johansen[1], Horst Rose [2], Martin Steiner[2], Gabriela Senti[1], Claudio Rhyner[3], Reto Crameri[3], and Thomas M. Kündig[1]

[1] Unit for Experimental Immunotherapy, Department of Dermatology, University Hospital of Zurich, Gloriastrasse 31, 8091 Zurich, Switzerland

[2] ImVisioN GmbH, Feodor-Lynen Strasse 5, 30625 Hannover, Germany

[3] Swiss Institute of Allergy and Asthma Research (SIAF), Obere Strasse 22, 7270 Davos, Switzerland

The project was partly funded by ImVisioN GmbH, Hannover, Germany. Work at SIAF was supported by the Swiss National Science Foundation (grant 310000-114634) and by the OPO-Pharma foundation, Zurich.

Allergy *in press*

ABSTRACT

Background: Current subcutaneous allergen-specific immunotherapy (SIT) leads to amelioration of IgE mediated allergy, but it requires numerous allergen injections over several years and is frequently associated with severe side effects. The aim of this study was to test whether modified recombinant allergens can improve therapeutic efficacy in SIT while reducing allergic side effects.

Methods: The major cat allergen Fel d 1 was fused to a TAT-derived protein translocation domain and to a truncated invariant chain for targeting the MHC class II pathway (MAT-Fel d 1). The immunogenicity was evaluated in mice, while potential safety issues were assessed by CAST using basophils from cat dander allergic patients.

Results: MAT-Fel d 1 enhanced induction of Fel d 1 specific IgG2a antibody responses as well as the secretion of IFN-γ and IL-2 from T cells. SIT of mice using the modified Fel d 1 provided stronger protection against anaphylaxis than SIT with unmodified Fel d 1, and MAT-Fel d 1 caused less degranulation of human basophils than native Fel d 1.

Conclusion: MAT-Fel d 1 allergen enhanced protective antibody and Th1 responses in mice, while reducing human basophil degranulation. Immunotherapy using MAT-Fel d 1 allergen has the potential to enhance SIT efficacy and safety, thus, shortening SIT. This should increase patient compliance and lower treatment costs.

INTRODUCTION

Allergen-specific immunotherapy (SIT) is the only causal treatment of allergies, has a long-lasting effect and can stop progression of the allergy to multiple sensitisations or to asthma. Subcutaneous SIT typically requires 30-80 injections during 3-5 years (Pichler et al., 2001) and bears a significant risk of allergic side effects including anaphylaxis. Hence, the number of injections and also the risk of allergic side effects should be reduced. Current efforts to enhance SIT focus on optimizing the allergen molecules (Karamloo et al., 2005; Larche, 2007; Saarne et al., 2005), the adjuvants (Johansen et al., 2005b; Santeliz et al., 2002), the route of administration (Barbey et al., 2004; Bohle et al., 2007; Razafindratsita et al., 2007), and also their dosage form, including use of particulate delivery systems, such as virus like (Kundig et al., 2006) or poly(lactide-co-glycolide) particles (Martinez Gomez et al., 2007).

Approaches to increase the safety of SIT include pre-treatment with anti-histamines or anti-IgE antibodies (Kopp et al., 2007). Safety can also be improved by disrupting IgE binding epitopes by heat denaturation (Johansen et al., 2005c), by chemical modifications to generate allergoids (García-Sellés J, 2003), using genetically engineered allergens with reduced IgE binding capacity (Karamloo et al., 2005; Saarne et al., 2005), or using synthetic peptides that do not bind IgE but stimulate T cell responses (Larche, 2007). However, reduced IgE binding has often been followed by reduced immunogenicity of the allergens (Wurtzen et al., 2007).

We have recently proposed a new concept for allergy vaccines based on targeting the MHC class II antigen presentation pathway (Crameri et al., 2007). These so-called modular antigen transporter (MAT) recombinant allergens consist of an allergen fused to a TAT-derived translocation peptide and to the first 110 amino-acids of the human invariant chain (Ii). The TAT peptide mediates cytoplasmic uptake of extracellular proteins (Fittipaldi and Giacca, 2005; Gump and Dowdy, 2007). Small molecules are believed to enter cells via electrostatic interactions in an energy-independent manner, whereas large molecules are taken up by energy-dependent macropinocytosis (Herce and Garcia, 2007). Early in biosynthesis, MHC class II αβ heterodimers assemble in the endoplasmic reticulum with an Ii trimer to form a nonameric complex (Roche et al., 1991). Ii binds to the MHC class II molecule and blocks the class II peptide binding groove until the MHC II-Ii complexes are transported to the endosomes where Ii is removed by proteolysis, thus permitting loading of

the groove with endosomal peptides (Hiltbold and Roche, 2002). Fusing the allergen to Ii therefore directly links the allergen with the class II pathway of antigen presentation.

In this study, we used Fel d 1 as a model allergen and tested the influence of the above described modifications on immune responses *in vivo*. To avoid potential differences in distribution and pharmacokinetics of the different proteins, the allergens were administered directly into the inguinal lymph nodes of mice (Johansen et al., 2005a).

METHODS

Allergens

Cat fur allergen extract was purchased from Stallergènes (Fresnes, France). rFel d 1 (19 kDa), TAT-Fel d 1 (21 kDa; with the HIV-TAT sequence GYGRKKRRQRRR) and MAT-Fel d 1 (34 kDa; with HIV-TAT plus amino acids 1-110 of the human invariant chain) were engineered on pQE30 expression vectors (Qiagen, Hilden, Germany) containing an N-terminal $[His]_6$-tag for protein purification and were produced in *Escherichia coli* as described previously (Crameri et al., 2007).

Immunisation protocols

CBA female mice (6-8 weeks; Harlan, Horst, The Netherlands) were immunised thrice with two-week intervals by intralymphatic (i.l.) injection (Johansen et al., 2005a) with 30 pmol of allergen, 90 μg of $Al(OH)_3$ (Alhydrogel 3%, Brenntag Biosector, Fredrikssund, Denmark) and saline (10 μl) into the inguinal lymph node or by subcutaneous (s.c.) injection with 300 pmol of allergen, 450 μg $Al(OH)_3$ and saline (50 μl). Moreover, the MAT-Fel d 1 immunisation regime was optimised by testing different doses and numbers of i.l. injections. Serum was prepared from tail vein blood and frozen at -20°C until analysed.

The therapeutic potential of the allergens was tested in mice sensitised by 6 weekly intraperitoneal (i.p.) injections of 1 μg cat fur allergen extract with 900 μg $Al(OH)_3$ in 100 μl. Subsequently, mice were desensitised with the different allergen molecules administered by i.l. injections as described above. For induction of anaphylaxis, sensitised mice were challenged with 20 μg of cat fur allergen extract in saline (50 μl i.p.). Body temperature was measured with a calibrated digital thermometer before and 30 minutes after the challenge.

Safety was assessed by challenging sensitised mice with 5 μM of the different Fel d 1 allergen molecules in saline as described above.

All animal experiments were approved by and performed according to the guidelines from the Swiss veterinary service authorities.

Antibody measurements

For antibody detection, 96-well microtitre plates (Nunc Maxisorb, Basel, Switzerland) were coated with 1 μg/ml cat fur allergen extract in carbonate buffer and incubated overnight at 4°C. After blocking the plates with 2.5% non-fat dry milk in PBS-0.05% Tween-20 (PBSTM), serial dilutions of individual sera in PBSTM were added to the plates for 2 h. The plates were then incubated with biotinylated goat anti-mouse IgG1 or IgG2a (BD Pharmingen, San Diego, CA) in PBSTM, followed by incubation with streptavidin-conjugated horseradish peroxidase (BD Pharmingen, San Diego, CA, USA). Finally, the plates were added the enzyme substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (Sigma-Aldrich, Buchs, Switzerland) in 1 M sodium dihydrogen phosphate and absorption read at 405 nm on a Model 550 Microplate reader (BioRad, Hercules, CA, USA). Unless otherwise specified, all incubations were done at room temperature and intercepted with PBST washes.

For detection of total IgE antibodies, plates were coated with 2 μg/ml of anti-mouse IgE capture antibody (BD Pharmingen) at 4°C overnight. As secondary reagent to bound mouse serum, biotinylated anti-mouse IgE (BD Pharmingen) was used at 2 μg/ml.

To determine the IgE binding capacity of the different Fel d 1 proteins, plates were coated with anti-mouse IgE (BD Pharmingen) as above, washed, blocked with PBSTM, and incubated 2 h with serum samples from sensitised mice. Then, cat fur allergen extract, rFel d 1, TAT-Fel d 1 or MAT-Fel d 1 were added at 0.6 μM and the plates incubated for another 90 min. Two different antibodies were then used for detection: anti-Histag-Biotin (Qiagen, Hombrechtikon, Switzerland) or anti-Fel d 1-Biotin (Indoors, Biotechnology, Warminster, United Kingdom) at 1/1000 dilution (90 min). The plates were developed using enzyme and substrate as described above.

Cytokine-secretion assay

Mice were immunised thrice by i.l. injection with 30 pmol of MAT-Fel d 1, TAT-Fel d 1 or rFel d 1, as described above. One week later, spleens were isolated and single-cell suspensions prepared. Erythrocytes were removed by lysis, and triplicates of $8x10^5$ cells/well were cultured with 10 μg/ml LoTox Fel d 1 (Indoors Biotechnology) or left unstimulated in 200 μl of supplemented IMDM. Supernatants were collected after 20 h for IL-2 determination and after 72 h for determination of IL-4, IL-10 and IFN-γ using the DuoSet ELISA from R&D Systems (Abingdon, United Kingdom).

Allergenicity in human leukocytes

Freshly withdrawn blood from ten patients with allergy to cat dander was prepared for analysis by the cellular allergen stimulation test (CAST-2000 ELISA, Bühlmann Laboratories AG, Schönenbuch, Switzerland). Leukocytes were isolated by dextran sedimentation and incubated with IL-3 together with cat fur allergen extract, rFel d 1 or MAT-Fel d 1 at different concentrations for 40 minutes. Samples were then frozen at -80°C until analysed for quantification of the leukotrienes released in the supernatant.

This single centre clinical study was performed between September and December 2005 with patients recruited from the allergy unit of the Zurich University Hospital. Inclusion criteria were a clear history of cat dander allergy documented by quantitative Fel d 1-specific IgE ImmunoCAP determinations (Phadia, Uppsala Sweden) and positive skin prick test (wheal diameter $\geq$ 3 mm^2). The study was approved by the local ethical review committee, performed according to GCP guidelines and the Declaration of Helsinki. All patients gave informed consent before entering the trial (clinicaltrials.gov Identifier: NCT00620880).

Statistics

To assess statistical variances data were analysed by non-parametric tests, i.e.Mann-Whitney U test or Kruskal-Wallis one-way analysis of variance with Dunn's multiple comparison test using shared p-values. The significance level was set at 95%.

RESULTS

MAT allergens enhanced IgG2a antibody responses in mice

All three recombinant Fel d 1 allergens induced high levels of cat allergen-specific IgG1 antibodies (Fig. 1a), independent on the route of administration. The IgG1 levels were lower for the mice injected with MAT-Fel d 1 i.l. as compared to the other i.l. injected proteins ($p<0.05$). The IgG2a responses (Fig. 1b) were significantly different between the three groups ($p<0.05$), having mice immunised with MAT-Fel d 1 or TAT-Fel d 1 higher IgG2a levels than rFel d 1. Moreover, the levels of IgG2a were higher upon i.l. than upon s.c. immunisation for all three proteins.

The ratio of IgG2a to IgG1 is a parameter for the relative strength of Th1 to Th2 immune responses. All ratios were higher for i.l. than for s.c. injections, indicating a preferential Th1 polarisation by the i.l. route (Fig. 1c). Immunisation of mice with MAT-Fel d 1 induced significantly higher IgG2a-to-IgG1 ratios as compared to rFel d 1 ($p<0.01$), both after s.c. and i.l. administration. All antibody responses were long-lasting, and the IgG2a-to-IgG1 ratios remained unchanged.

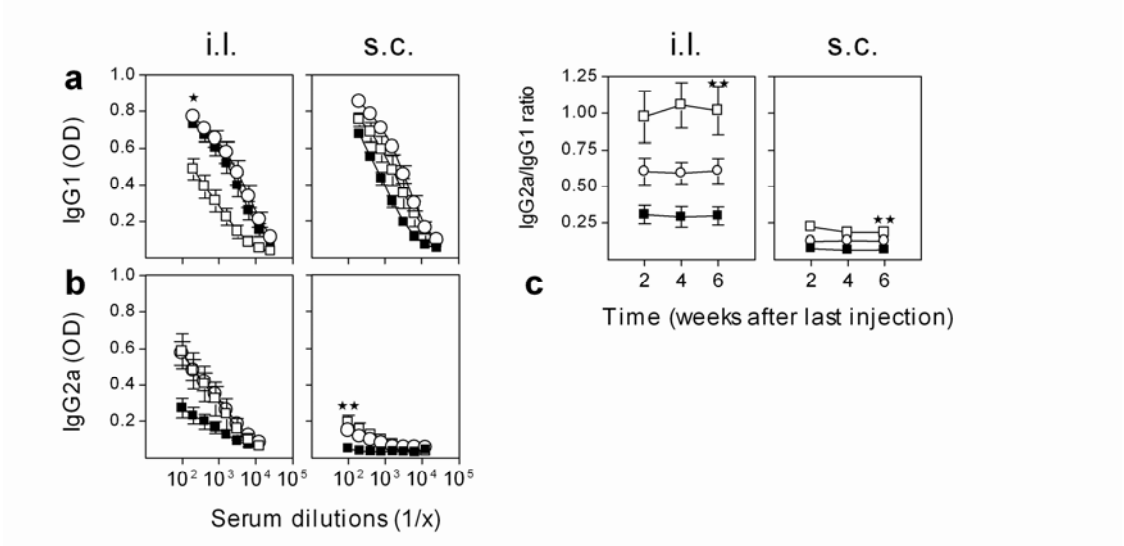

Figure 1. Cat allergen-specific serum antibodies of mice immunised thrice with 30 pmol intralymphatically (i.l.) or 300 pmol subcutaneously (s.c.) of recombinant Fel d 1 (■), TAT-Fel d 1 (○) or MAT-Fel d 1 (□). Six weeks after the last injection sera were analysed by ELISA for IgG1 **(a)** or IgG2a **(b)**. Symbols represent means ± SEM (n = 8). The ratio of IgG2a to IgG1 **(c)** was calculated for the indicated time points at 1/400 serum dilution as a parameter for the Th1/Th2 balance. Statistical analyses were done using the Kruskal-Wallis test with Dunn's multiple comparison posttest (** $p < 0.01$; * $p < 0.05$), differences shown in the graphs are applied to rFel d 1 and MAT-Fel d 1.

Three intralymphatic immunisations with 0.1 µg MAT-Fel d 1 were sufficient to induce both IgG1 and IgG2a antibodies (Fig. 2a). The immune responses significantly increased for both subclasses at 1 µg ($p<0.05$), which represented an optimum, as no further increase of the response was observed at 10 µg. At a dose of 1 µg, optimal immunisation regimen was achieved with three i.l. injections (Fig. 2b). One injection was inferior ($p<0.05$), while four injections did not further improve the response.

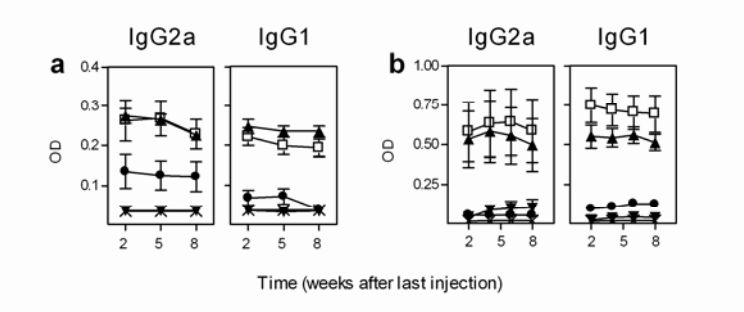

Figure 2. Cat allergen-specific IgG2a and IgG1 during the optimisation of the immunisation regime. (a) Mice were immunised thrice intralymphatically with 0 (X), 0.01 (▼), 0.1 (●), 1 (▲) or 10 µg (□) of MAT-Fel d 1. Sera were analysed at 2, 5 and 8 weeks after the last injection. (b) Mice received from either no injections (X), or 1 (▼), 2 (●), 3 (▲) or 4 (□) intralymphatic injections of MAT-Fel d 1, respectively. Antibody levels were measured at 2, 4, 6 and 8 weeks after the last injection. For both experiments antibody levels are expressed as mean optical densities at 1/100 (a) or 1/400 (b) sera dilutions ± SEM (n = 5).

Vaccination with MAT-Fel d 1 allergen increased Th1 cytokine production

Upon re-stimulation of splenocytes from mice immunised with the different Fel d 1 allergens, only cells from MAT-Fel d 1 immunised mice secreted significant levels of IL-2 ($p<0.05$) (Fig. 3a). Immunisation with MAT-Fel d 1 also increased secretion of IFN-γ (Fig. 3b) as compared to TAT-Fel d 1 ($p<0.05$) and rFel d 1. In contrast, IL-4 (Fig. 3c) and IL-10 (Fig. 3d) secretion was found to be stronger upon immunisation with rFel d 1. Stimulation of cells from untreated mice did not induced detectable levels of the mentioned cytokines (not shown).

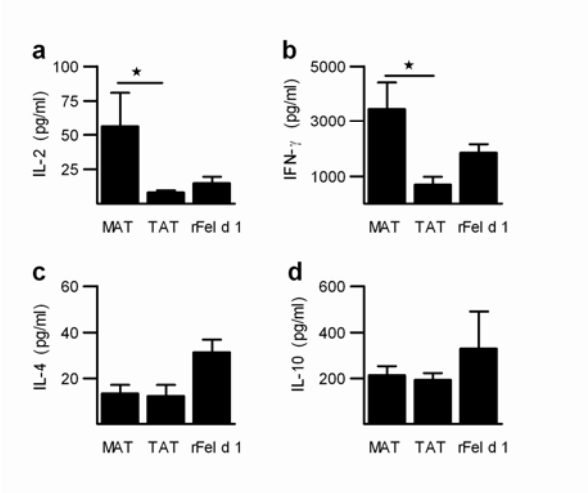

Figure 3. Cytokine secretion *in vitro.* Splenocytes from mice immunised with MAT-Fel d 1, TAT- Fel d 1 or rFel d 1 were analysed for secretion of IL-2 (a), IFN-γ (b), IL-4 (c) and IL-10 (d) after *in vitro* re-stimulation with 10 µg/ml LoTox Fel d 1. The results show the cytokine concentrations in supernatants after subtraction of spontaneous secretion from unstimulated splenocytes as determined by ELISA. Statistical differences are indicated when $p < 0.05$ as analysed by the Kruskal-Wallis test with Dunn's multiple comparison posttest (n =4).

Immunotherapy with modified Fel d 1 enhanced protection against anaphylaxis

Mice sensitised to cat fur allergen extract had high levels of serum IgE and IgG1, but no detectable IgG2a (Fig. 4a; time point zero). Upon desensitisation with three i.l. injections of different allergens, MAT-Fel d 1 induced the highest IgG2a levels, followed by TAT-Fel d 1, rFel d 1 and cat fur extract. Four weeks after completed immunotherapy, mice were challenged with a high-dose of cat fur allergen extract and subsequently monitored for anaphylaxis. Mice treated with MAT-Fel d 1 showed stronger protection against anaphylaxis than mice treated otherwise (Fig. 4b), however, it was only significantly different from the cat fur allergen extract treated group ($p<0.05$) but not compared to rFel d 1 treated group.

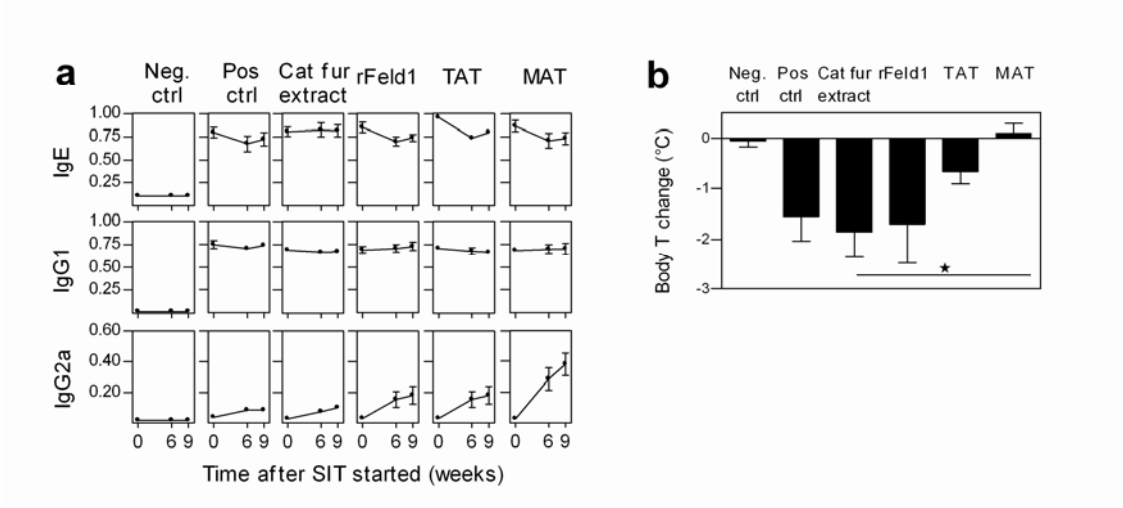

Figure 4. Murine anaphylaxis model. (a) Cat allergen-specific IgG1 and IgG2a antibodies and total IgE were analysed in sera from sensitised mice before SIT (time point 0), as well as 6 and 9 weeks after the first SIT injection. The negative control group consisted of naive mice, and the positive control was a group of sensitised mice that did not receive SIT. (b) Four weeks after the last SIT injection, mice were challenge with 20 µg of cat fur allergen extract i.p. and the body temperature drop was measured before and 30 minutes after the challenge. Data were analysed by the Kruskal-Wallis test with Dunn's multiple comparison posttest (* $p < 0.05$) (n = 5).

Modified allergens did not induce anaphylaxis in sensitised mice

To assess the safety of modified recombinant allergens in SIT, their capacity to induce anaphylaxis in sensitised mice was measured. After the last of six sensitisation injections with cat fur allergen extract, all animals had comparable high levels of Fel d 1 specific IgG1 and IgE, whereas IgG2a was not detectable (not shown). Mice were then challenged with the different recombinant Fel d 1 allergens or with cat fur allergen extract (Fig. 5). Three out five mice challenged with rFel d 1 and five out of five challenged with cat fur allergen extract experienced anaphylaxis while none of the mice challenged with MAT-Fel d 1 or TAT-Fel d 1 showed a notable drop in body temperature.

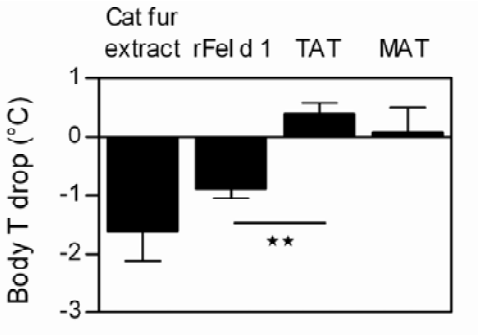

Figure 5. *In vivo* safety. Mice were sensitised with cat fur allergen extract and 3 weeks after the last injections challenged with the pure extract or the different Fel d 1 proteins. Body temperature was measured before and 30 minutes after the challenge. Results are illustrated as means ± SEM (n=5). Differences between the three recombinant proteins were analysed by the Kruskal-Wallis test with Dunn's multiple comparison posttest (**$p<0.01$).

Reduced degranulation of human basophils after incubation with MAT-Fel d 1

Further evaluation of the potential safety of MAT-Fel d 1 in human immunotherapy was performed by testing the allergen-induced degranulation of human basophils from cat fur allergic patients in CAST-ELISA. While the second lowest concentration of cat fur allergen extract or rFel d 1 caused strong basophil degranulation and leukotriene release, a 100-fold higher concentration of MAT-Fel d 1 allergen was required to induce comparable basophil degranulation (Fig. 6).

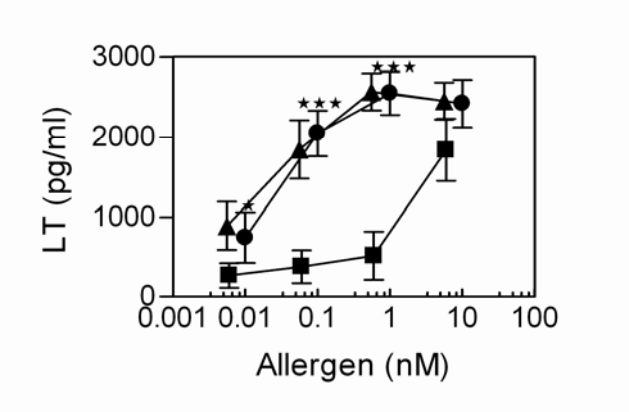

Figure 6. CAST ELISA assay. Blood from 10 cat allergic patients was used for measuring the leukotriens (LT) released after incubation with MAT-Fel d 1 (■), rFel d 1 (▲) or cat fur allergen extract (●) at 4 different concentrations. Results are illustrated as means ± SEM and statistical comparison between MAT-Fel d 1 and rFel d 1 was analysed by the Mann-Whitney U test (*** $p < 0.001$, * $p < 0.05$).

DISCUSSION

Although SIT offers medical advantages over symptomatic treatments of IgE mediated allergies, merely 3-4% of allergic patients choose to undergo SIT, mainly due to the long treatment duration and the frequently associated allergic side effects. For use in SIT it would therefore be highly desirable to have allergens that enhance efficacy and at the same time reduce allergic side effects, i.e., to render SIT faster and safer. In the present study, different modified recombinant Fel d 1 allergens were tested for their therapeutic potential in SIT. The allergens were in part modified by fusing them to a protein translocation sequence to improve cellular uptake and a truncated invariant chain for targeting to the MHC class II pathway of antigen presentation (Crameri et al., 2007).

SIT aims at regulating allergen-specific immune responses, e.g. to trigger Th1 cells as well as neutralising antibody responses. The presence of Th1 cytokines such as IFN-γ inhibits the production of IL-4 and thereby prevents the IL-4 mediated IgE-switch of B cells. IgE enhances the expression of high affinity IgE receptors (FcεRI) (Kawakami and Galli, 2002), hence, allergy is self promoting. Our study revealed that mice immunised with MAT-Fel d 1 had higher levels of IFN-γ and lower levels of IL-4 than mice immunised with the unmodified Fel d 1 allergen. Furthermore, the enhanced production of Th1 assisted antibodies, IgG2a in mice (Johansen et al., 2005c) and IgG1 and IgG4 antibodies in humans (Till et al., 2004), has been reported after SIT. In line with this, immunotherapy of sensitised mice using MAT-Fel d 1 induced higher IgG2a levels and conferred better protection against a challenge with a high dose of the cat fur allergen extract.

In addition to being more efficient in stimulating Th1 immune responses, our study revealed that MAT-Fel d 1 also reduced basophil degranulation and leukotriene release by a 100-fold when compared to unmodified Fel d 1 or the cat fur allergen extract. This potentially increased safety profile of MAT-Fel d 1 was confirmed in sensitised mice that showed no anaphylaxis after a high-dose challenge with MAT-Fel d 1 or TAT-Fel d 1. This increased safety of the modified allergens could be explained by a reduced IgE-binding capacity. However, while MAT-Fel d 1 indeed showed reduced IgE binding (not shown), TAT-Fel d 1 did not. Therefore, the reduced allergic reactivity observed in mice of the TAT allergen and maybe also in part of the MAT allergen is rather the result of a more rapid cellular uptake (Brooks et al., 2005; Gump and Dowdy, 2007) that prevents the

allergen from binding to FcεRI on mast cells and basophils. In conclusion, these results demonstrated that MAT- Fel d 1 not only increased SIT efficacy but also safety.

Over the last ten years, several studies have reported enhanced presentation of antigens through the MHC class-II pathway by different strategies that involved the Ii (Bischof et al., 2001; Carstens et al., 2000; Kallinteris et al., 2006). For instance, the so called Ii-Key peptides, consist of a short fragment of the human Ii (hIi aa77-92 or aa77-80) fused to an antigenic peptide (Kallinteris et al., 2006). The Ii-Key binds to an allosteric site of the MHC-II molecules on the cell surface and the antigenic peptide binds to the epitope binding groove, increasing the loading of MHC class II molecules and, therefore, enhancing the immunogenicity of the loaded antigen. The MAT allergens used in our study had a longer sequence of the hIi (aa 1-110). This targets the MHC class-II molecules in the endoplasmatic reticulum (Crameri et al., 2007) where it occupies the peptide binding groove, and thereby avoids interaction with other antigens. The complex formed by the MAT-allergen and the MHC class-II molecule is directed through the Golgi to the endo/lysosomal compartment where the Ii can be degraded together with the fused allergen. Now, the allergenic peptides can be loaded to the MHC class-II molecules and be transported to the cell surface (Bonehill et al., 2005). As a result, MAT allergens increase the efficiency of allergen presentation, which furthermore result in an increase in the effective allergen dose available for stimulation of protective immune responses. Indeed, high allergen doses suppress IgE production while low doses are supportive of its production (Ruedl et al., 2000), and human immunotherapy with high allergen doses is more efficient than using lower allergen doses (Powell et al., 2007). While this strategy in theory could be advantageously used for SIT, the risk of allergic adverse events limits the possibility of increasing the therapeutic allergen dose and explains the need for a controlled dose-escalation during the early phase of SIT. However, the safety profile as well as the cell-penetration and MHC-targeting properties of MAT allergens may allow SIT with high allergen doses, making such allergens highly attractive in human therapy.

This study, as well as other studies in mice (Johansen et al., 2005a) and humans (manuscript in preparation), also demonstrated that intralymphatic allergen administration may strongly improve SIT efficacy and allow lower allergen doses to be used, which again would improve safety as compared to subcutaneous SIT. Alternative routes of allergen administration, e.g. sublingual (SLIT), oral and intranasal applications (Barbey et al., 2004; Bohle et al., 2007; Razafindratsita et al., 2007) have been introduced as a means to improve

SIT. However, as most proteins have short half-lives, most of such administered allergens are degraded before exerting their immunotherapeutic effects. For this reason, intralymphatic injections, in which the allergen is administered directly into a subcutaneous lymph node, i.e., to the site of action of the immune system, represent an advantage over other administration routes.

In conclusion, our data demonstrated that the two main problems of current SIT, its relatively low efficiency requiring a high number of injections, as well as the risk of allergic reactions, could both be improved by using MAT allergens.

Acknowledgements

The authors thank Mrs. María J. Pena Rodríguez for help with the ELISA measurements, Dr. Nicole Graf for help with statistics, Dr. Susanne Haug for withdrawing the patients' blood, Dr. Andrea Hofmann and Prof. Adriano Aguzzi for helpful discussions.

Author contributions

Pal Johansen and me designed research, analysed data and wrote the manuscript; Martin Steiner and Horst Rose designed research and revised the manuscript; Claudio Rhyner. synthesised the recombinant allergens; Reto Crameri provided recombinant allergens and corrected the manuscript; Gabriela Senti designed the clinical trial; Thomas M. Kündig initiated and designed research and wrote the manuscript.

REFERENCES

Barbey, C., Donatelli-Dufour, N., Batard, P., Corradin, G. and Spertini, F. (2004) Intranasal treatment with ovalbumin but not the major T cell epitope ovalbumin 323-339 generates interleukin-10 secreting T cells and results in the induction of allergen systemic tolerance. *Clin Exp Allergy*, **34**, 654-662.

Bischof, F., Wienhold, W., Wirblich, C., Malcherek, G., Zevering, O., Kruisbeek, A.M. and Melms, A. (2001) Specific treatment of autoimmunity with recombinant invariant chains in which CLIP is replaced by self-epitopes. *Proceedings of the National Academy of Sciences*, **98**, 12168-12173.

Bohle, B., Kinaciyan, T., Gerstmayr, M., Radakovics, A., Jahn-Schmid, B. and Ebner, C. (2007) Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. *J Allergy Clin Immunol*, **120**, 707-713.

Bonehill, A., Heirman, C. and Thielemans, K. (2005) Genetic approaches for the induction of a CD4+ T cell response in cancer immunotherapy. *J Gene Med*, **7**, 686-695.

Brooks, H., Lebleu, B. and Vives, E. (2005) Tat peptide-mediated cellular delivery: back to basics. *Adv Drug Delivery Rev*, **57**, 559.

Carstens, C., Newman, D.K., Bohlen, H., Konig, A. and Koch, N. (2000) Invariant chains with the class II binding site replaced by a sequence from influenza virus matrix protein constrain low-affinity sequences to MHC II presentation. *Int. Immunol.*, **12**, 1561-1568.

Crameri, R., Fluckiger, S., Daigle, I., Kundig, T. and Rhyner, C. (2007) Design, engineering and in vitro evaluation of MHC class-II targeting allergy vaccines. *Allergy*, **62**, 197-206.

Fittipaldi, A. and Giacca, M. (2005) Transcellular protein transduction using the Tat protein of HIV-1. *Adv Drug Deliv Rev*, **57**, 597-608.

García-Sellés J, P.A., Funes E, Pagán JA, López JD, Negro JM, et al. (2003) Clinical efficacy and safety of a depigmented and glutaraldehyde polymerized therapeutic vaccine of Parietaria judaica. *Allergol Immunopathol (Madr)*, **31**, 63-69.

Gump, J.M. and Dowdy, S.F. (2007) TAT transduction: the molecular mechanism and therapeutic prospects. *Trends Mol Med*, **13**, 443-448.

Herce, H.D. and Garcia, A.E. (2007) Molecular dynamics simulations suggest a mechanism for translocation of the HIV-1 TAT peptide across lipid membranes. *Proc Natl Acad Sci U S A*, **104**, 20805-20810.

Hiltbold, E.M. and Roche, P.A. (2002) Trafficking of MHC class II molecules in the late secretory pathway. *Curr Opin Immunol*, **14**, 30-35.

Johansen, P., Haffner, A.C., Koch, F., Zepter, K., Erdmann, I., Maloy, K., Simard, J.J., Storni, T., Senti, G., Bot, A., Wuthrich, B. and Kundig, T.M. (2005a) Direct intralymphatic injection of peptide vaccines enhances immunogenicity. *Eur J Immunol*, **35**, 568-574.

Johansen, P., Senti, G., Martinez Gomez, J.M., Storni, T., von Beust, B.R., Wuthrich, B., Bot, A. and Kundig, T.M. (2005b) Toll-like receptor ligands as adjuvants in allergen-specific immunotherapy. *Clin Exp Allergy*, **35**, 1591-1598.

Johansen, P., Senti, G., Martinez Gomez, J.M., Wuthrich, B., Bot, A. and Kundig, T.M. (2005c) Heat denaturation, a simple method to improve the immunotherapeutic potential of allergens. *Eur J Immunol*, **35**, 3591-3598.

Kallinteris, N.L., Lu, X., Blackwell, C.E., von Hofe, E., Humphreys, R.E. and Xu, M. (2006) Ii-Key/MHC class II epitope hybrids: a strategy that enhances MHC

class II epitope loading to create more potent peptide vaccines. *Exp Opin Biol Ther*, **6**, 1311-1321.

Karamloo, F., Schmid-Grendelmeier, P., Kussebi, F., Akdis, M., Salagianni, M., von Beust, B.R., Reimers, A., Zumkehr, J., Soldatova, L., Housley-Markovic, Z., Muller, U., Kundig, T., Kemeny, D.M., Spangfort, M.D., Blaser, K. and Akdis, C.A. (2005) Prevention of allergy by a recombinant multi-allergen vaccine with reduced IgE binding and preserved T cell epitopes. *Eur J Immunol*, **35**, 3268-3276.

Kawakami, T. and Galli, S.J. (2002) Regulation of mast-cell and basophil function and survival by IgE. *Nat Rev Immunol*, **2**, 773-786.

Kopp, M.V., Stenglein, S., Kamin, W., Friedrichs, F., von Berg, A., Zielen, S., Hamelmann, E., Wahn, U. and Kuehr, J. (2007) Omalizumab (Xolair) in children with seasonal allergic rhinitis: Leukotriene release as a potential in vitro parameter to monitor therapeutic effects. *Pediatr Allergy Immunol*, **18**, 523-527.

Kundig, T.M., Senti, G., Schnetzler, G., Wolf, C., Prinz Vavricka, B.M., Fulurija, A., Hennecke, F., Sladko, K., Jennings, G.T. and Bachmann, M.F. (2006) Der p 1 peptide on virus-like particles is safe and highly immunogenic in healthy adults. *J Allergy Clin Immunol*, **117**, 1470-1476.

Larche, M. (2007) Update on the current status of peptide immunotherapy. *J Allergy Clin Immunol*, **119**, 906-909.

Martinez Gomez, J.M., Fischer, S., Csaba, N., Kundig, T.M., Merkle, H.P., Gander, B. and Johansen, P. (2007) A Protective Allergy Vaccine Based on CpG- and Protamine-Containing PLGA Microparticles. *Pharm Res*, **24**, 1927-1935.

Pichler, C.E., Helbling, A. and Pichler, W.J. (2001) Three years of specific immunotherapy with house-dust-mite extracts in patients with rhinitis and asthma: significant improvement of allergen-specific parameters and of nonspecific bronchial hyperreactivity. *Allergy*, **56**, 301-306.

Powell, R.J., Frew, A.J., Corrigan, C.J. and Durham, S.R. (2007) Effect of grass pollen immunotherapy with Alutard SQ on quality of life in seasonal allergic rhinoconjunctivitis. *Allergy*, **62**, 1335-1338.

Razafindratsita, A., Saint-Lu, N., Mascarell, L., Berjont, N., Bardon, T., Betbeder, D., Van Overtvelt, L. and Moingeon, P. (2007) Improvement of sublingual immunotherapy efficacy with a mucoadhesive allergen formulation. *J Allergy Clin Immunol*, **120**, 278-285.

Roche, P.A., Marks, M.S. and Cresswell, P.J. (1991) Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature*, **354**, 392-394.

Ruedl, C., Bachmann, M.F. and Kopf, M. (2000) The antigen dose determines T helper subset development by regulation of CD40 ligand. *Eur J Immunol*, **30**, 2056-2064.

Saarne, T., Kaiser, L., Gronlund, H., Rasool, O., Gafvelin, G. and van Hage-Hamsten, M. (2005) Rational design of hypoallergens applied to the major cat allergen Fel d 1. *Clin Exp Allergy*, **35**, 657-663.

Santeliz, J.V., Nest, G.V., Traquina, P., Larsen, E. and Wills-Karp, M. (2002) Amb a 1-linked CpG oligodeoxynucleotides reverse established airway hyperresponsiveness in a murine model of asthma. *J Allergy Clin Immunol*, **109**, 455-462.

Till, S.J., Francis, J.N., Nouri-Aria, K. and Durham, S.R. (2004) Mechanisms of immunotherapy. *J Allergy Clin Immunol*, **113**, 1025-1034; quiz 1035.

Wurtzen, P.A., Lund, L., Lund, G., Holm, J., Millner, A. and Henmar, H. (2007) Chemical modification of birch allergen extract leads to a reduction in allergenicity as well as immunogenicity. *Int Arch Allergy Immunol*, **144**, 287-295.

CHAPTER 4

A protective allergy vaccine based on CpG- and protamine-containing PLGA microparticles

A protective allergy vaccine based on CpG- and protamine-containing PLGA microparticles

Julia M. Martínez Gómez[1], Stefan Fischer[2], Noèmi Csaba[2], Thomas M. Kündig[1], Hans P. Merkle[2], Bruno Gander[2], and Pål Johansen[1]

[1]Unit for Experimental Immunotherapy, Department of Dermatology, University Hospital Zurich, Zurich, Switzerland

[2]Drug Delivery and Formulation, Institute of Pharmaceutical Sciences, ETH Zurich, Zurich, Switzerland

Published in: Pharmaceutical Research (2007) 24(10):1927-35

ABSTRACT

PURPOSE: Allergen-specific immunotherapy (SIT) requires dozens of subcutaneous injections over three to five years in order to control IgE-mediated hypersensitivity, which is a T-helper 2 (Th2)-associated pathology. This study investigates the use of poly(lactide-co-glycolide) (PLGA) microparticles combined with immunostimulatory oligodeoxynucleotide (CpG) as well as protamine in SIT.

METHODS: We prepared microparticle formulations with the major allergen of bee venom, phospholipase A2 (PLA2), and analyzed the effect of co-encapsulated or admixed CpG in both naïve and bee venom allergic mice.

RESULTS: Mice immunized with microparticles containing only PLA2 induced weak antibody responses. In contrast, the combination with CpG resulted in strong PLA2-specific antibody responses. The presence of CpG was required for the induction of the Th1-associated isotype IgG2a, and the titers of IgG2a in sensitized mice correlated with a better protection against an allergen challenge. The effect of CpG was further strengthened when protamine was co-encapsulated for complexation of CpG.

CONCLUSIONS: This study shows that allergen-specific immunotherapy with a PLGA-based allergen-delivery system in combination with CpG enhanced the induction of protective IgG2a immune responses. This may improve SIT compliance and shorten its duration.

INTRODUCTION

Approximately 20% of the population in developed countries suffers from IgE-mediated type-I hypersensitivity. The clinical manifestations of this type of allergy are rhino-conjunctivitis, asthma or even life-threatening anaphylactic reactions. These symptoms are all initiated by the degranulation of mast cells and basophils when their surface-bound IgE molecules are cross-linked through the binding of the allergen. Although symptomatic treatments are available, the only treatment with long-lasting effect for allergic patients is subcutaneous allergen-specific immunotherapy (SIT). During SIT, gradually increasing doses of the allergen are injected subcutaneously. This shifts the immune response against the allergen from a predominantly Th2-type response (IgE and IL-4) towards a Th1-like immune response (IgG and IFN-γ). The risk of allergic side effects is currently a major disadvantage of SIT. This has motivated the study of safer allergens such as oligopeptides derived from allergens, or recombinant proteins with reduced IgE-binding capacity (Alexander et al., 2002; Kussebi et al., 2005; Larche, 2002). A second significant disadvantage of SIT is the high cost with a total of 30-80 injections administered over years (Durham et al., 1999). A simplified SIT with a reduced number of injections would, therefore, be highly advantageous, as it would improve patient compliance and provide socio-economic benefits. The most frequently common adjuvants in SIT are aluminum salts, being known to favor Th2 responses (including IgE production). Aluminum salts can cause local granuloma formation at the injection site, but otherwise have a good safety record in SIT. Nevertheless, many efforts are directed towards the use of new adjuvants that favor Th1 responses (Wheeler and Woroniecki, 2001).

Biodegradable materials such as poly(lactide-co-glycolide) (PLGA) represent a potential alternative for controlled delivery of allergens in SIT (Jilek et al., 2004). PLGA preparations can deliver proteins over prolonged periods of time and induce protective immunity after a single subcutaneous injection (Peyre et al., 2004; Peyre et al., 2003). In addition, they may also comprise adjuvants or other immune-regulating compounds, e.g. Th1-triggering compounds.

Major candidate adjuvants that shift the immune responses towards Th1 are derived from pathogens. The so-called pathogen-associated molecular patterns (PAMPs) are recognized by receptors (Toll-like receptors, TLRs) on antigen presenting cells (APCs) (Akira and Sato, 2003). TLR binding may activate nuclear factor-κB, which directs the

secretion of cytokines, chemokines and co-stimulatory molecules important for efficient Th1 immune responses. Bacterial DNA containing cytosine-guanine rich immunostimulatory sequences (CpG) have been recognized to bind to TLR-9 and to trigger strong Th1 responses (Krieg, 2002; Krieg et al., 1995).

In this study, we tested PLGA microparticles in combination with CpG for their use in subcutaneous SIT against the major bee venom allergen phospholipase A2 (PLA2). In mice, isotype switching to IgG2a strongly depends on Th1 CD4 T-cell help. Therefore, the capacity of a vaccine to trigger IgG2a antibodies was used as an important indicator for the screening of appropriate formulations. We found that CpG strongly enhanced the immune response and was a prerequisite for the induction of IgG2a antibody responses. This effect was more pronounced when the CpG was co-encapsulated rather than admixed to PLA2-containing MP. The best prophylactic and therapeutic immune responses were obtained with PLGA preparations that contained both co-encapsulated CpG and protamine.

MATERIALS AND METHODS

Materials

Purified phospholipase A_2 (PLA2) from bee venom was purchased from Sigma-Aldrich (Buchs, Switzerland). Bee venom extract from ALK-Abelló was purchased through Trimedal (Bruttisellen, Switzerland). Aluminum hydroxide (Alhydrogel 2%) was purchased from Brenntag Biosector (Fredrikssund, Denmark), and phosphorothioate-modified CpG oligodeoxynucleotide 1668pt (5'-TCC-ATG-ACG-TTC-CCT-GAC-GTT-3') was synthesized by Microsynth (Balgach, Switzerland). We used a 35 kDa poly(lactide-co-glycolide) (PLGA 50:50) with uncapped end-groups (Resomer RG503H) from Boehringer-Ingelheim (Ingelheim, Germany). Salmine sulfate from salmon sperm (protamine sulfate with four arginine residues at the C-terminus) and poly(vinyl alcohol) (PVA, Mowiol 4-88) were obtained from Fluka (Buchs, Switzerland).

Microparticle preparation

PLGA microparticles (MP) were made by microextrusion-based w/o/w-solvent extraction using a static multilamination type micromixer (*Institut für Mikrotechnik Mainz GmbH*, Mainz, Germany), as previously described (Freitas et al., 2003), with slight modifications. Three different formulations were prepared. For one formulation, 2 mg of PLA2 were dissolved in 100 µl water and emulsified by ultrasonication with 7 ml PLGA dissolved in dichloromethane (5%, w/w). For the others either 4 mg PLA2 or a mixture of 4 mg PLA2 and 8 mg protamine were dissolved in 1.6 ml water containing 1.6 µmol CpG; this aqueous phase was emulsified with 12 ml of a 5% PLGA solution in dichloromethane. After extrusion in the micromixer, the suspension of MP was collected in a borosilicate glass beaker containing an aqueous solution of 0.5% (w/w) PVA for solvent extraction. The particles were gently stirred using a magnetic rod and kept in a laminar air flow for 30 min for further solvent removal and hardening of the particles. Finally, the particles were collected on a 0.8 µm pore-sized mixed cellulose ester membrane filter (Schleicher & Schuell, Dassel, Germany) and dried at 20 mbar and room temperature for 24 h.

Particle size determination

Microparticle size distributions were determined by laser diffraction using a Mastersizer X (Malvern Instrument, Malvern, UK) after suspending approximately 1 mg of

dried MP in distilled water or in an aqueous solution of 1% lecithin. The average cumulative undersize distributions (D_{10}, D_{50} and D_{90}) were determined on a volume basis.

Zeta potential measurements

The surface charge of the MP was determined by zeta potential measurement using a Zetasizer 3000 HSA from Malvern Instrument (Malvern, UK). Duplicates of approximately 0.2 mg of dried MP were re-suspended in 2 ml of a solution of 1 mM KCl (pH 7.6). Alternatively, the MP were re-suspended in 1% lecithin and then diluted with 2 ml of 1 mM KCl. Mean values of zeta potential were calculated from triplicates of each sample.

Determination of allergen content and integrity in the microparticles

The extraction of allergen from the MP for the analysis of its content and integrity was undertaken as previously described (Gebrekidan et al., 2000). Triplicates of approx. 7 mg particles were dissolved in 0.5 ml chloroform by vortexing for three minutes. PLA2 was then extracted from the organic phase by mixing with 0.2 ml Tris-HCl buffer and subsequent centrifugation at 3000 rpm for 3 minutes. The PLA2 concentration in the aqueous supernatant was analyzed by an ELISA inhibition assay. In detail, 60 µl of the Tris-HCl phase containing the extracted PLA2 were mixed in a 96-well plate with 60 µl of diluted (1:25) human anti-PLA2 serum in phosphate-buffered saline containing 0.05% Tween20 and 2.5% skimmed dry milk (PBSTM). In parallel, two-fold dilutions of PLA2 were mixed with sensitized serum, and established as a standard for quantification. Positive (only serum) and negative (only PBSTM) controls were also included. After 2 hours of incubation at 37°C, 100 µl of each sample were transferred to an ELISA plate that was pre-coated with 5 µg/ml PLA2 at 4°C overnight and subsequently blocked with PBSTM at room temperature for 1 hour. The samples were then incubated at 37 °C for 2 hours. Subsequently, the plate was washed and incubated with 100 µl of a 1:1000 dilution of horseradish peroxidase-conjugated anti-human IgG (BD Biosciences Pharmingen, San Diego, CA) at room temperature for 1 hour. The plate was washed and developed with 100 µl of the enzyme substrate 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (Sigma-Aldrich) in 1 M sodium dihydrogen phosphate. After 30 min, the absorption was read at 405 nm. The loading efficiency of PLA2 in the MP was calculated relative to the theoretical maximum loading value.

To assess the integrity of the allergen during MP preparation and storage, PLA2 from the various MP formulations was extracted with chloroform (as described above), loaded

under reduced conditions on SDS-PAGE and run at 180 V for 50 min. Following electrophoresis, the gel was stained with the silver-staining method as previously described (Nesterenko et al., 1994).

In vitro release of PLA2

Accurately weighed PLA2-loaded MP (20 mg) were dissolved in 3.4 ml PBS containing 0.2% BSA and incubated in a shaker at 37°C for 63 days. At different time points, the samples were centrifuged at 3500 rpm for 10 minutes. Aliquots of the supernatants were analyzed by inhibition ELISA to quantify the released PLA2. The dissolution medium was replaced with fresh PBS/BSA after each sampling.

Immunization of mice

In a preliminary set of experiments, the dose of PLA2 contained in PLGA MP necessary to induce solid immune responses was determined. For dose finding, mice were immunized twice at an interval of 28 days with MP formulations (MP-PLA2, MP-PLA2-CpG-protamine) containing either 1 µg or 5 µg of PLA2, or with 1 µg of PLA2 adsorbed on aluminum hydroxide. Blood was collected on days 28, 56 and 84. The sera were frozen and kept at -20°C until analyzed by ELISA.

The immunogenicity of PLA2-containing PLGA formulations was tested in 6-10 week old female CBA/J mice. Mice were primed with 1 µg PLA2 entrapped in the different PLGA formulations (Table 1). Prior to injection, the MP were re-suspended using a 1% aqueous lecithin solution (Epikuron 200, Degussa, Hamburg, Germany) as a wetting agent and injection vehicle. For the MP-PLA2 + CpG formulation, 3.1 nmol CpG per dose was admixed to the MP-PLA2 prior to injection. As a reference, one group of mice received the same amount of PLA2 adsorbed for 1 hour to 0.9 mg aluminum hydroxide (PLA2 + aluminum hydroxide). Approximately 95% of the PLA2 was adsorbed to aluminum hydroxide (data not shown). All mice were boosted with the same regime after 28 days. Serum was prepared from clotted blood taken on days 28, 55 and 84 and frozen at -20°C until analyzed by ELISA.

To evaluate the therapeutic potential of PLA2-containing preparations in allergic mice, the animals were sensitized by 6 weekly intraperitoneal injections of 0.3 µg bee venom allergen extract adsorbed on 1 mg aluminum hydroxide in PBS. Three weeks later (time point 0), desensitization (SIT) was initiated with different MP preparations containing 1 µg PLA2 or, alternatively, 5 µg PLA2 adsorbed on aluminum hydroxide. The treatment was

repeated after 28 days with the same dose and, after 111 days, with 5 μg PLA2 in all formulations (MP and aluminum hydroxide). Blood was taken on days 0, 28, 55, 111 and 139 after the first SIT injection. The sera were frozen and kept at -20°C until analyzed by ELISA. For induction of anaphylactic responses, immunized mice were challenged intraperitoneally with 15 μg PLA2 in saline, and the rectal temperature was measured with a calibrated digital thermometer before and 30 min after the challenge.

All animal experiments were performed according to the guidelines of the veterinary authorities of the Canton of Zurich.

Antibody determination by enzyme-linked immunosorbent assay

For detection of PLA2 antibodies, microtitre 96-well plates (Nunc Maxisorb) were coated with 100 μL of 5 μg/mL PLA2 in buffered carbonate (pH 9.4) and incubated at 4°C overnight. Plates were washed with PBS-0.05% Tween 20 (PBST) and blocked with 150 μl of 2.5% PBSTM for 1 hour. After washing, serial dilutions of individual sera in 100 μL PBSTM were incubated in the plates for 2 hours. Subsequently, the plates were washed and incubated with 1 μg/mL biotinylated goat anti-mouse IgG1 or IgG2a in 100 μL PBSTM for 2 hours. After washing and incubation with 100 μL of a 1:1000 dilution of streptavidin-conjugated horse-radish peroxidase for 1 hour, the plates were washed and developed with 100 μL enzyme substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (Sigma-Aldrich) in 1 M sodium dihydrogen phosphate. After 20 min, the endpoint absorption was measured at 405 nm. For detection of specific IgE antibodies, plates were coated with 2 μg/ml of anti-mouse IgE capture antibody. As secondary reagent for binding to mouse serum, an in-house biotinylated PLA2 was used at 3 μg/ml. Unless specified, all antibodies were from BD Biosciences Pharmingen, and all incubations were done at room temperature.

RESULTS

Microparticle characterization

Three different PLGA formulations were prepared by a recently developed microextrusion based process (Fischer et al., 2006; Freitas et al., 2003). The size of the particles increased slightly depending on the number of encapsulated components (Table 1). While 50% of the MP-PLA2-CpG-protamine particles were smaller than 8.2 μm, half the MP-PLA2 particles were smaller than a 4.41 μm. The 90% cumulative undersize values were comparable for all tested formulations, i.e., 20.1 μm (MP-PLA2-CpG) to 26.3 μm (MP-PLA2-CpG-protamine). The re-suspension of the particles in 1% lecithin resulted in an apparent increase in size of the MP-PLA2-CpG formulation but not for the other formulations (not shown).

Table 1. Zeta potential and PLA2 content of the tested microparticle (MP) preparations.

	Size distribution (μm) D_{10} / D_{50} / D_{90}	Zeta potential (mV) w/o or w lecithin	PLA2 content (μg/mg MP)	PLA2 loading efficiency (%)
MP-PLA2	1.2 / 4.4 / 24.9	-7.3 ± 0.3 -12.3 ± 1.6	0.84	29
MP-PLA2 + CpG	1.2 / 4.4 / 24.9	-7.3 ± 0.5 -9.7 ± 0.5	0.84	29
MP-PLA2-CpG	1.3 / 5.6 / 20.1	-9.1 ± 1.8 -12.5 ± 0.9	1.55	33
MP-PLA2-CpG-protamine	2.4 / 8.2 / 26.3	-9.6 ± 1.8 -13.7 ± 1.6	0.95	22

The MP-PLA2 + CpG corresponds to the MP-PLA2 preparation, but contains CpG that was admixed prior to injection; therefore, the PLA2 content of the two preparations are the same. The particle size distribution is indicated as D_{10}, D_{50} and D_{90} cumulative undersizes.

The zeta potential of all MP formulations was negative and changed only marginally upon admixture of CpG (from -7.5 mV for MP-PLA2 to -7.3 mV for MP-PLA2 + CpG), or upon co-encapsulation of CpG (-9.5 mV for MP-PLA2-CpG), or else upon co-encapsulation of both CpG and protamine (-9.6 mV for MP-PLA2-CpG-protamine) (Table 1). When the particles were re-suspended in lecithin, the zeta potentials decreased significantly in all formulations ($p<0.01$; un-paired, two-sided Student's t-test).

The experimentally determined PLA2 content in the MP ranged from 0.84 μg/mg (MP-PLA2) to 1.55 μg/mg (MP-PLA2-CpG) (Table 1). The corresponding microencapsulation efficiencies were in the range of 22% to 33%.

The analysis of extracted PLA2 from the different MP formulations by SDS-PAGE produced a unique band for PLA2 at 16 kDa (Fig. 1), which does not suggest any cleavage of the allergen during preparation and storage (6-12 months) of the MP. Furthermore, extracted PLA2 maintained its antigenicity as confirmed by inhibition ELISA with a polyclonal human antiserum (data not shown), suggesting that the protein structure remained intact during the MP preparation and storage.

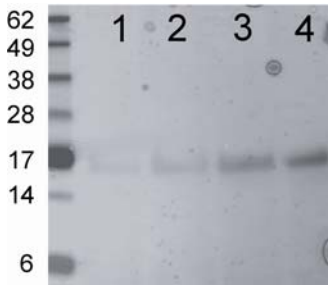

Figure 1. Silver-stained SDS-PAGE of PLA2 extracted from the different microparticle formulations. Seeblue Plus was used as a marker. Lane 1 corresponded to MP-PLA2, lane 2 to MP-PLA2-CpG, lane 3 to MP-PLA2-CpG-protamine and the last lane (4) was loaded with 1 μg of purified PLA2 as a positive control. For all the other lanes, maximal loading volumes were used.

The *in vitro* release of PLA2 from all formulations was triphasic and followed the same pattern (Fig. 2). An initial burst release during the first 24 hours was followed by a dormant period over two weeks and a second release phase lasting more than seven weeks. While the release of PLA2 from the protamine-containing formulation was complete over this time period, the release from the protamine- and CpG-free formulation (MP-PLA2) reached only 63%.

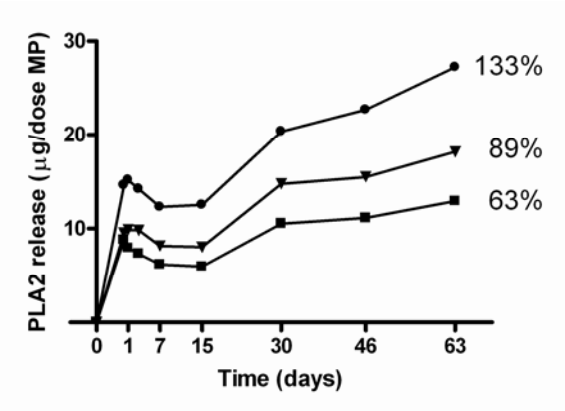

Figure 2. Allergen *in vitro* release profile of the different MP formulations. PLA2 release at different time points as measured by inhibition ELISA and expressed in microgram per dose of MP used for the experiment and total release in percentage. MP-PLA2 (■), MP-PLA2-CpG (▼) and MP-PLA2-CpG-protamine (●).

Immunogenicity

We first determined the dose of microencapsulated PLA2 required to induce a specific immune response in mice after subcutaneous administration of two different PLGA MP preparations. Mice were immunized with 1 µg or 5 µg of microencapsulated PLA2 in MP-PLA2 or MP-PLA2-CpG-protamine and boosted 28 days later with the same doses. As a control, one group of mice was primed and boosted with 1 µg PLA2 adsorbed on aluminum hydroxide. As illustrated in Figure 3, 5 µg PLA2 in MS-PLA2 induced high levels of IgG1 while the 1 µg PLA2 dose induced only weak IgG1 responses and no detectable IgG2a antibodies. The CpG- and protamine-containing particles were strongly immunogenic for both IgG1 and IgG2a induction both at 1 µg and 5 µg PLA2, and a single administration was sufficient for seroconversion in the latter group. Aluminum-adsorbed PLA2 induced only IgG1 antibodies and boosting was required.

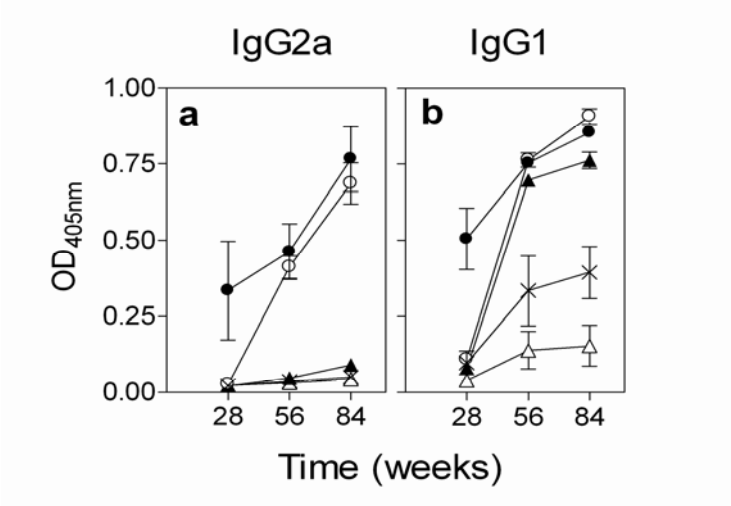

Figure 3. Dose-dependant immunogenicity of PLA2-containing microparticles. Mice (n=3) were immunized on days 0 and 28 with 1 µg (empty symbols) or 5 µg (filled symbols) of PLA2 contained in MP-PLA2 (triangles), MP-PLA2-CpG-protamine (circles), or with 1 µg of PLA2 adsorbed on $Al(OH)_3$ (×). The sera were obtained at days 28, 56 and 84 and analyzed by ELISA for anti-PLA2 IgG2a (a) and IgG1 (b). The absorbances at 1/160 serum dilution were chosen as representative for each time point.

Based on these results, we tested the immunogenicity of four different PLGA MP preparations containing 1 µg of PLA2 (Table 1). Mice received two injections 28 days apart, and the PLA2-specific antibodies were measured 28, 55 and 84 days after the first injection. In line with the results above, a single administration of the CpG-free formulation (MP-PLA2) induced little or no detectable anti-PLA2 antibodies by day 28 (Figure 4). At this time point, only the formulation MP-PLA2-CpG-protamine induced some IgG1, but no IgG2a antibodies. A second injection on day 28 did not notably increase the antibody titers

of mice that had received MP-PLA2, unless CpG was admixed prior to injection (MP-PLA2 + CpG). The co-encapsulation of CpG (MP-PLA2-CpG) further increased this response, which was additionally enhanced when both CpG and protamine were co-encapsulated (MP-PLA2-CpG-protamine). Maximum antibody titers for both IgG subclasses were observed one month after the second injection (day 55), after which time the antibody levels slightly decreased or remained stable during the next month (day 84). The IgG2a levels induced by the two MP preparations with encapsulated CpG (MP-PLA2-CpG and MP-PLA2-CpG-protamine), but not with admixed CpG (MP-PLA2 + CpG), were significantly higher than those induced by PLA2 adsorbed on aluminum hydroxide ($p<0.05$ for the MP-PLA2-CpG and $p<0.001$ for the MP-PLA2-CpG-protamine, as analyzed by a two-way ANOVA with Bonferroni posttest). Also, the quality of the total immune response was different between the immunogenic PLGA preparations and the aluminum adsorbed control vaccine. The latter induced an IgG1 (Th2) polarized antibody response, while the particles shifted the response towards an IgG2a (Th1), as evident from the IgG2a-to-IgG1 ratios (Figure 4c). Neither the PLGA preparations nor the control group (aluminum hydroxide) induced detectable PLA2-specific IgE antibodies (data not shown).

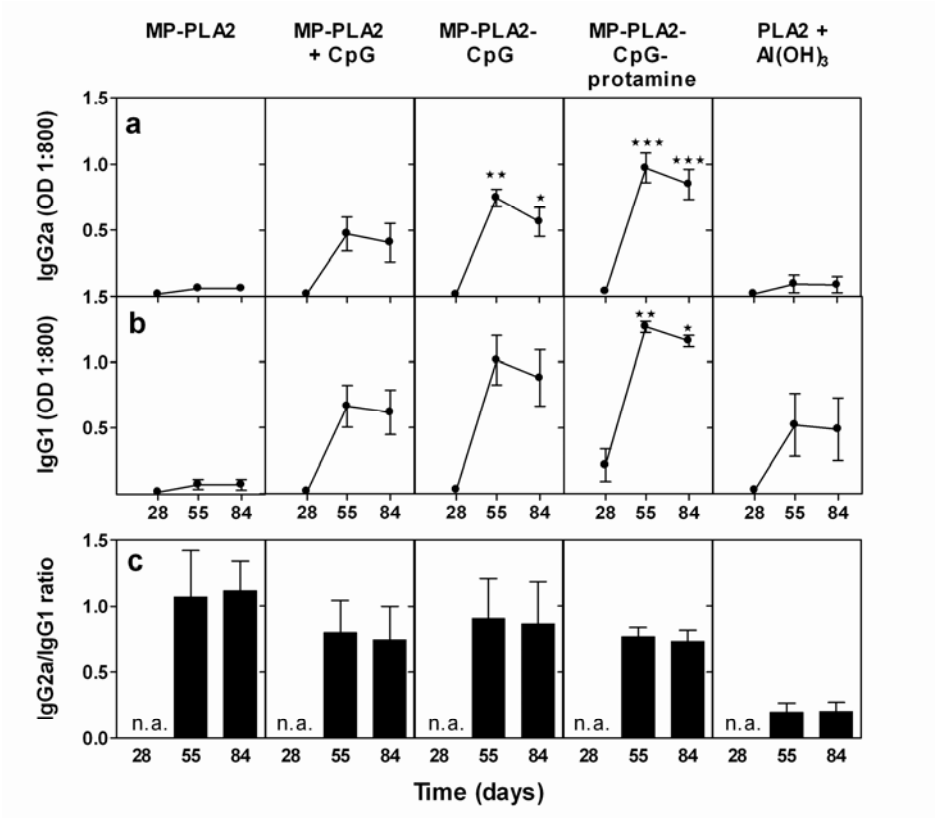

Figure 4. Isotype profiles of anti-PLA2 serum antibodies. CBA/J mice were vaccinated on days 0 and 28 with 1 μg PLA2 in different microparticle formulations, as indicated, or adsorbed on aluminum hydroxide as reference (n=4). Blood was collected at days 28, 55 and 84 and the sera analyzed by ELISA for anti-PLA2

IgG2a (a) and IgG1 (b). Ratio of IgG2a to IgG1 was calculated at 1/800 serum dilution and only values above seroconversion (absorbance higher or equal than that of a negative serum plus three standard deviations) are plotted (c). Data were analyzed by a two-way ANOVA with Bonferroni posttest. Significant differences are indicated as compared to the control group (*** $p<0.001$; ** $p<0.01$; * $p<0.05$).

Allergen-specific immunotherapy

To evaluate the therapeutic potential of the different MP formulations, we used a murine anaphylaxis model. Mice were sensitized by six weekly intraperitoneal injections of bee venom allergen extract adsorbed on aluminum hydroxide. Three weeks after the last sensitization injection, SIT was initiated by subcutaneous injections with different PLGA formulations containing 1 μg PLA2 or with 5 μg PLA2 adsorbed on aluminum hydroxide (Fig. 5).

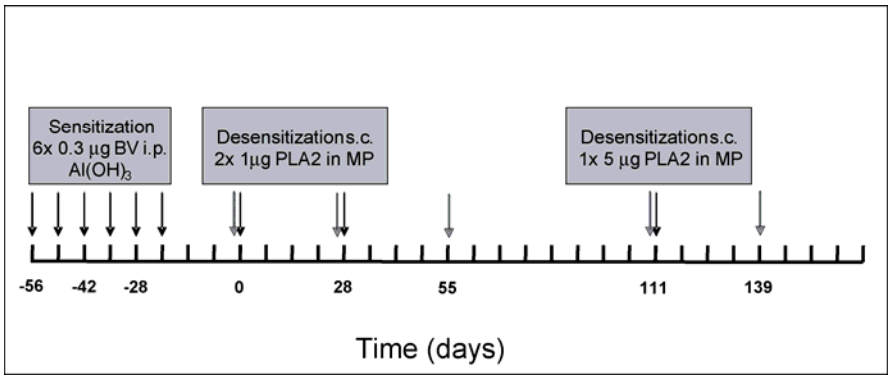

Figure 5. Schematic illustration of the therapeutic immunization protocol. Black arrows represent injections and grey arrows represent bleedings.

As evident from Figure 6a, two injections with 1 μg of microencapsulated PLA2 were not sufficient to induce detectable titers of PLA2-specific IgG2a antibodies in the sensitized mice. Therefore, the animals were boosted at day 111 after the first SIT injection with the same MP preparations, but at the higher dose of 5 μg PLA2. This increased the levels of allergen-specific IgG2a significantly compared to the pre-boosting titers. The two formulations with encapsulated CpG induced significantly stronger ($p<0.05$, as analyzed by a two-way ANOVA with Bonferroni posttest) IgG2a responses than those induced by the control vaccine with aluminum hydroxide. Finally, to assess the functional efficacy of the different treatments, mice were challenged with a high dose of PLA2, and their body temperature monitored before and after the challenge. Mice treated with MP containing CpG had the smallest body temperature drop (Fig. 6b), being significantly lower than that observed in the untreated control group ($p<0.05$ for MP-PLA2-CpG and $p<0.01$ for MP-PLA2-CpG-protamine, as analyzed by a one-way ANOVA with Dunnet's Multiple comparison test). In fact, IgG2a responses correlated with protection against anaphylaxis

(p=0.02, correlation analyzed by the Pearson test). The higher the IgG2a titers, the lower the temperature drop (Fig. 6c).

The levels of bee-venom specific of IgE and IgG1 increased similarly for all groups during the sensitization period and remained unchanged during SIT. For all groups, PLA2-specific IgG1 increased comparably during both sensitization and SIT (data not shown).

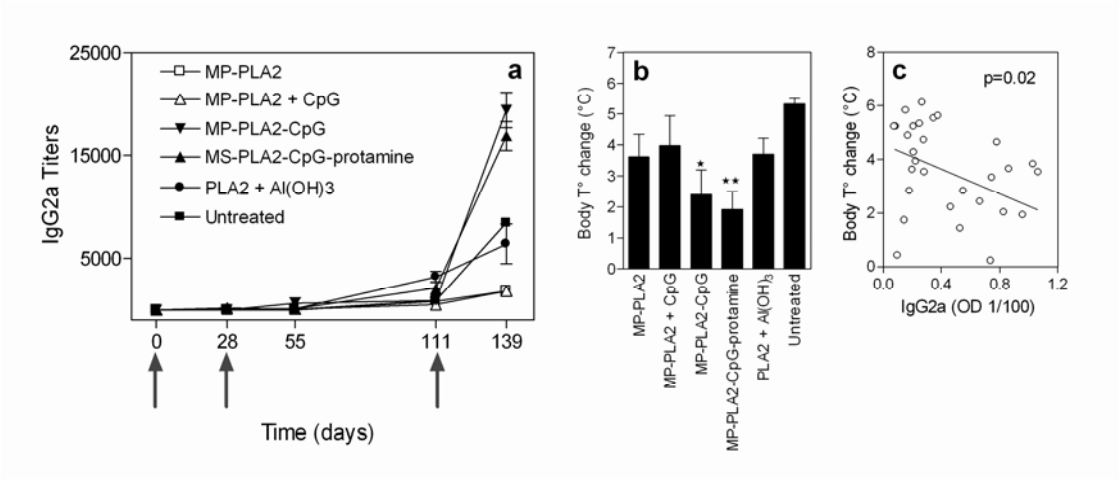

Figure 6. Therapeutic potential of PLA2-containing microparticles. (a) Sensitized mice as described in the Materials and Method section were therapeutically vaccinated with MP-PLA2 (□), MP-PLA2 + CpG (Δ), MP-PLA2-CpG (▼), MP-PLA2-CpG-protamine (▲), PLA2 adsorbed on Al(OH)3 (●), or left untreated (■). IgG2a titers were defined as the inverse of the highest dilution reaching an absorbance equal or higher than that of a negative serum plus three standard deviations and expressed as geometric means ± standard error (n=5). Similar levels of IgG1 and IgE were seen for all groups treated (not shown). (b) Induction of anaphylaxis responses against PLA2 in desensitized mice. After desensitization, the mice were challenged with 15 µg of purified PLA2, and the body temperature was measured before and 30 minutes after the challenge. Values are expressed as body temperature difference before and after. Significant differences are indicated as compare to the untreated group (** $p<0.01$; * $p<0.05$). (c) Correlation between the level of protective antibodies and body temperature change. The IgG2a absorbance from the last time point was plotted versus the body temperature change in challenged mice. The correlation was analyzed by the Pearson test, which assumes that the data is sampled from a Gaussian population, with a 95% confidence interval.

DISCUSSION

Allergen-specific immunotherapy (SIT) aims to shift the balance between Th2 and Th1 responses in favor of the latter. The consequences are an increase in Th1-related antibodies (IgG4 in humans, IgG2a in mice) and cytokines (IFN-γ) (Durham et al., 1996; Gehlhar et al., 1999), a reduction in Th2-associated IgE and IL-4 (Secrist et al., 1993), and an increase in allergen-specific suppressive activity through regulatory CD25-positive CD4 T cells and IL-10 (Nouri-Aria et al., 2004; Till et al., 2004). However, current SIT is costly and requires years of allergen injections. As the allergen dose cannot be increased due to allergic side effects, concepts to enhance the efficacy of SIT have focused on creating recombinant allergens with less allergic side effect or on the developing of new adjuvants and delivery systems. So far, only adjuvants based on aluminum and calcium salts have been approved for SIT. Both compounds preferentially stimulate Th2-polarized antibody responses (Bousquet et al., 1998), but T-cell responses are poor.

Biodegradable and biocompatible polymeric MP are particularly interesting candidates for allergy vaccines, because they release proteins over prolonged periods of time (Ying Men et al., 1996) and, thereby, reduce the need for frequent booster injections. Since entrapped allergen would be less accessible for binding IgE on the surface of mast cells and basophils, the encapsulation of allergens may also protect the allergic patient from undesired allergic side effects. Due to their particulate nature and small size, MP also have intrinsic adjuvant properties and may be combined with immunostimulatory agents to trigger distinct pathways of immunological reactions.

Oligodeoxynucleotides rich in cytosine-guanine immunostimulatory sequences (CpG) are a prototype of Th1 response inducers. CpG stimulates TLR9 on APCs (Hemmi et al., 2000). It has been shown that CpG can restore imbalanced Th2-Th1 responses in allergic mice (Banerjee et al., 2004; Johansen et al., 2005a), and that the conjugation of a ragweed allergen to CpG improved the therapeutic efficacy in patients with allergic rhinitis (Creticos et al., 2006).

In the present study, we combined CpG with allergen-containing PLGA MP and evaluated the immunogenicity and therapeutic potential of the formulations in a murine model of allergy. The results suggested that co-encapsulation of allergen and CpG in PLGA MP can have important benefits for application in SIT. CpG lowered the dose-threshold for induction of allergen-specific immune responses. This paralleled a study that

showed that PLGA MP containing a streptococcus antigen and CpG elicited significantly higher antigen-specific antibody responses when compared with the same vaccine without CpG (Hunter et al., 2001). Moreover, only CpG-containing preparations induced IgG2a antibodies, suggesting that a potent allergy vaccine based on PLGA MP should also contain CpG or an equivalent Th1-triggering adjuvant. The therapeutic model confirmed this finding, by showing that CpG-containing MP preparations protected mice against induced anaphylaxis better than CpG-free preparations.

Despite this clear adjuvant effect of CpG, oligodeoxynucleotides have relatively short half-life *in vivo* due to degradation by nucleases (Agrawal et al., 1995; Farman and Kornbrust, 2003). For *in vivo* use, therefore, they have been stabilized by phosphorothioate-modification of the oligodeoxynucleotide backbone (Agrawal et al., 1995; Sester et al., 2000). Alternatively, CpG has been conjugated to stabilizing compounds (Lee et al., 2000; Park et al., 2001) or packaged into virus-like particles (Storni et al., 2004). For the same purpose, we tested whether the PLGA MP-based allergy vaccine could be further improved by adding protamine to the formulation, as a putative stabilizer for DNA. The experiments revealed that the immunogenicity of the vaccine was indeed further enhanced when protamine was co-encapsulated. The induction of both IgG1 and IgG2a isotypes increased, with a slightly stronger increase of the latter isotype. This resulted in better protection against anaphylaxis, as compared to all other PLA2 vaccine preparations tested.

Protamine is a cationic polypeptide that is known to stabilize DNA in the haploid phase of spermatogenesis. Pharmaceutically, it has been used to preserve plasmid DNA for gene transfer (Li and Huang, 1997) and to avoid degradation of oligodeoxynucleotides (Gonzalez Ferreiro et al., 2003). Besides direct stabilization of CpG, the improved performance of the protamine-containing formulations could also be a secondary result of more efficient encapsulation of CpG due to complexation with protamine. Indeed, CpG adsorption was found to be stronger on protamine-containing particles than on protamine-free particles and the release of CpG was strongly sustained with the former particles (S. Fischer, unpublished data). Moreover, due to the neutralization of the negative charges on CpG by the positive protamine as well as the increased size of the protamine-CpG complex, the complex is expected to have a higher affinity for the polymer matrix than CpG alone. This should allow a more delayed release of the CpG, which again would allow CpG and the allergen to reach the same APCs. Such a synchronized delivery of danger

signals (adjuvant; so-called signal 0 and 2) and allergen (so-called signal 1) is one important criterion for optimal stimulation of long-lasting immune responses (Schijns, 2000). The importance of such synchronized allergen and CpG delivery was further underlined by the fact that admixed CpG, released by a faster kinetics than the encapsulated allergen, induced weaker antibody responses and less protection than co-encapsulated allergen and CpG. Experiments with MP formulations that contained co-encapsulated PLA2 and protamine, but no CpG, performed similarly to the MP-PLA2 formulation (results not shown). This implies that co-encapsulated protamine exerted its effect through stabilization and improved encapsulation of CpG and not due to a direct immune regulatory or stimulatory effect.

One of the major concerns about encapsulation of therapeutic proteins and antigens is the risk of degradation or structural changes during the process. This has so far limited the clinical use of MP. In our study, the encapsulation of PLA2 did not seem to affect its integrity or immunogenicity. Nonetheless, some disruption of the molecule may not be as damaging for allergens as it may for other proteins. In fact, many of the new approaches in allergy treatment use modified allergens with low IgE binding capacity, e.g., recombinant proteins (Crameri and Rhyner, 2006; Wild et al., 2007), chemically cross-linked (Bousquet et al., 1990) or heat-denaturated allergens (Johansen et al., 2005b; Kim et al., 2002).

To the best of our knowledge, this is the first report where the potential of PLGA MP with co-encapsulated allergen and CpG has been studied *in vivo* for use in allergen-specific immunotherapy. The preparations were strongly immunogenic in mice and the Th1-triggering effect of CpG was further improved by co-encapsulation of protamine. Importantly, this correlated with a better protection against anaphylaxis in bee venom allergic mice. Therefore, due to the increased efficacy and the reduced risk of allergic side effects, CpG- and allergen-containing PLGA MP represent a most attractive strategy for improving the conventional long-lasting and costly SIT.

Acknowledgements

The authors thank Mrs. María J. Pena Rodríguez for help with ELISA measurements, Mrs. Nathalie Schlegel and Dr. Nicole Graf for English editions, and Prof. Adriano Aguzzi for helpful discussions.

Author contributions

This paper is the result of a fruitful collaboration with the Institute of Drug Formulation and Delivery, from Pharmaceutical Sciences, ETH Zurich.

Together with Stefan Fischer, I prepared the different microparticle formulation at the ETH. I performed all animal experiment and analyzed the data. After the paper got reviewed, Noémi Csaba helped me to further characterize the microparticles *in vitro*.

Pål Johansen took part in the experimental design of the project and guided me through the writing of the manuscript. Bruno Gander and Thomas Kündig helped designing the experiments and corrected the manuscript. Hans Peter Merkle revised the manuscript.

REFERENCES

Agrawal, S., Temsamani, J., Galbraith, W. and Tang, J. (1995) Pharmacokinetics of antisense oligonucleotides. *Clin Pharmacokinet*, **28**, 7-16.

Akira, S. and Sato, S. (2003) Toll-like receptors and their signaling mechanisms. *Scand J Infect Dis*, **35**, 555-562.

Alexander, C., Kay, A.B. and Larche, M. (2002) Peptide-based vaccines in the treatment of specific allergy. *Curr Drug Targets Inflamm Allergy*, **1**, 353-361.

Banerjee, B., Kelly, K.J., Fink, J.N., Henderson, J.D., Jr., Bansal, N.K. and Kurup, V.P. (2004) Modulation of airway inflammation by immunostimulatory CpG oligodeoxynucleotides in a murine model of allergic aspergillosis. *Infect Immun*, **72**, 6087-6094.

Bousquet, J., Hejjaoui, A., Soussana, M. and Michel, F.B. (1990) Double-blind, placebo-controlled immunotherapy with mixed grass-pollen allergoids. IV. Comparison of the safety and efficacy of two dosages of a high-molecular-weight allergoid. *J Allergy Clin Immunol*, **85**, 490-497.

Bousquet, J., Lockey, R. and Malling, H.J. (1998) Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol*, **102**, 558-562.

Crameri, R. and Rhyner, C. (2006) Novel vaccines and adjuvants for allergen-specific immunotherapy. *Current Opinion in Immunology*, **18**, 761-768.

Creticos, P.S., Schroeder, J.T., Hamilton, R.G., Balcer-Whaley, S.L., Khattignavong, A.P., Lindblad, R., Li, H., Coffman, R., Seyfert, V., Eiden, J.J., Broide, D. and the Immune Tolerance Network, G. (2006) Immunotherapy with a Ragweed-Toll-Like Receptor 9 Agonist Vaccine for Allergic Rhinitis. *N Engl J Med*, **355**, 1445-1455.

Durham, S.R., Walker, S.M., Varga, E.M., Jacobson, M.R., O'Brien, F., Noble, W., Till, S.J., Hamid, Q.A. and Nouri-Aria, K.T. (1999) Long-term clinical efficacy of grass-pollen immunotherapy. *N Engl J Med*, **341**, 468-475.

Durham, S.R., Ying, S., Varney, V.A., Jacobson, M.R., Sudderick, R.M., Mackay, I.S., Kay, A.B. and Hamid, Q.A. (1996) Grass pollen immunotherapy inhibits allergen-induced infiltration of CD4+ T lymphocytes and eosinophils in the nasal mucosa and increases the number of cells expressing messenger RNA for interferon-gamma. *J Allergy Clin Immunol*, **97**, 1356-1365.

Farman, C.A. and Kornbrust, D.J. (2003) Oligodeoxynucleotide studies in primates: antisense and immune stimulatory indications. *Toxicol Pathol*, **31 Suppl**, 119-122.

Fischer, S., Foerg, C., Ellenberger, S., Merkle, H.P. and Gander, B. (2006) One-step preparation of polyelectrolyte-coated PLGA microparticles and their functionalization with model ligands. *Journal of Controlled Release*, **111**, 135.

Freitas, S., Walz, A., Merkle, H.P. and Gander, B. (2003) Solvent extraction employing a static micromixer: a simple, robust and versatile technology for the microencapsulation of proteins. *J Microencapsul*, **20**, 67-85.

Gebrekidan, S., Woo, B.H. and DeLuca, P.P. (2000) Formulation and in vitro transfection efficiency of poly (D, L-lactide-co-glycolide) microspheres containing plasmid DNA for gene delivery. *AAPS PharmSciTech*, **1**, E28.

Gehlhar, K., Schlaak, M., Becker, W. and Bufe, A. (1999) Monitoring allergen immunotherapy of pollen-allergic patients: the ratio of allergen-specific IgG4 to IgG1 correlates with clinical outcome. *Clin Exp Allergy*, **29**, 497-506.

Gonzalez Ferreiro, M., Crooke, R.M., Tillman, L., Hardee, G. and Bodmeier, R. (2003) Stability of polycationic complexes of an antisense oligonucleotide in rat small

intestine homogenates. *European Journal of Pharmaceutics and Biopharmaceutics*, **55**, 19.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K. and Akira, S. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature*, **408**, 740.

Hunter, S.K., Andracki, M.E. and Krieg, A.M. (2001) Biodegradable microspheres containing group B Streptococcus vaccine: Immune response in mice. *American Journal of Obstetrics and Gynecology*, **185**, 1174.

Jilek, S., Walter, E., Merkle, H.P. and Corthesy, B. (2004) Modulation of allergic responses in mice by using biodegradable poly(lactide-co-glycolide) microspheres. *J Allergy Clin Immunol*, **114**, 943-950.

Johansen, P., Senti, G., Martinez Gomez, J.M., Storni, T., von Beust, B.R., Wuthrich, B., Bot, A. and Kundig, T.M. (2005a) Toll-like receptor ligands as adjuvants in allergen-specific immunotherapy. *Clin Exp Allergy*, **35**, 1591-1598.

Johansen, P., Senti, G., Martinez Gomez, J.M., Wuthrich, B., Bot, A. and Kundig, T.M. (2005b) Heat denaturation, a simple method to improve the immunotherapeutic potential of allergens. *Eur J Immunol*, **35**, 3591-3598.

Kim, M.J., Lee, J.W., Yook, H.S., Lee, S.Y., Kim, M.C. and Byun, M.W. (2002) Changes in the antigenic and immunoglobulin E-binding properties of hen's egg albumin with the combination of heat and gamma irradiation treatment. *J Food Prot*, **65**, 1192-1195.

Krieg, A.M. (2002) CpG motifs in bacterial DNA and their immune effects *Annual Review of Immunology*, **20**, 709-760.

Krieg, A.M., Yi, A.-K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretzky, G.A. and Klinman, D.M. (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature*, **374**, 546.

Kussebi, F., Karamloo, F., Rhyner, C., Schmid-Grendelmeier, P., Salagianni, M., Mannhart, C., Akdis, M., Soldatova, L., Markovic-Housley, Z., Von Beust, B.R., Kundig, T., Kemeny, D.M., Blaser, K., Crameri, R. and Akdis, C.A. (2005) A major allergen gene-fusion protein for potential usage in allergen-specific immunotherapy. *J Allergy Clin Immunol*, **115**, 323-329.

Larche, M. (2002) Anti-T-cell strategies in the treatment of allergic disease. *Allergy*, **57 Suppl 72**, 20-23.

Lee, S.W., Song, M.K., Baek, K.H., Park, Y., Kim, J.K., Lee, C.H., Cheong, H.K., Cheong, C. and Sung, Y.C. (2000) Effects of a hexameric deoxyriboguanosine run conjugation into CpG oligodeoxynucleotides on their immunostimulatory potentials. *J Immunol*, **165**, 3631-3639.

Li, S. and Huang, L. (1997) In vivo gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. *Gene Therapy*, **4**, 891.

Nesterenko, M.V., Tilley, M. and Upton, S.J. (1994) A simple modification of Blum's silver stain method allows for 30 minute detection of proteins in polyacrylamide gels. *J Biochem Biophys Methods*, **28**, 239-242.

Nouri-Aria, K.T., Wachholz, P.A., Francis, J.N., Jacobson, M.R., Walker, S.M., Wilcock, L.K., Staple, S.Q., Aalberse, R.C., Till, S.J. and Durham, S.R. (2004) Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. *J Immunol*, **172**, 3252-3259.

Park, Y., Chang, Y.S., Lee, S.W., Cho, S.Y., Kim, Y.K., Min, K.U., Kim, Y.Y., Cho, S.H. and Sung, Y.C. (2001) The enhanced effect of a hexameric deoxyriboguanosine run conjugation to CpG oligodeoxynucleotides on protection against allergic asthma. *J Allergy Clin Immunol*, **108**, 570-576.

Peyre, M., Fleck, R., Hockley, D., Gander, B. and Sesardic, D. (2004) In vivo uptake of an experimental microencapsulated diphtheria vaccine following sub-cutaneous immunisation. *Vaccine*, **22**, 2430-2437.

Peyre, M., Sesardic, D., Merkle, H.P., Gander, B. and Johansen, P. (2003) An experimental divalent vaccine based on biodegradable microspheres induces protective immunity against tetanus and diphtheria. *J Pharm Sci*, **92**, 957-966.

Schijns, V.E. (2000) Immunological concepts of vaccine adjuvant activity. *Curr Opin Immunol*, **12**, 456-463.

Secrist, H., Chelen, C.J., Wen, Y., Marshall, J.D. and Umetsu, D.T. (1993) Allergen immunotherapy decreases interleukin 4 production in CD4+ T cells from allergic individuals. *J Exp Med*, **178**, 2123-2130.

Sester, D.P., Naik, S., Beasley, S.J., Hume, D.A. and Stacey, K.J. (2000) Phosphorothioate backbone modification modulates macrophage activation by CpG DNA. *J Immunol*, **165**, 4165-4173.

Storni, T., Ruedl, C., Schwarz, K., Schwendener, R.A., Renner, W.A. and Bachmann, M.F. (2004) Nonmethylated CG motifs packaged into virus-like particles induce protective cytotoxic T cell responses in the absence of systemic side effects. *J Immunol*, **172**, 1777-1785.

Till, S.J., Francis, J.N., Nouri-Aria, K. and Durham, S.R. (2004) Mechanisms of immunotherapy. *J Allergy Clin Immunol*, **113**, 1025-1034; quiz 1035.

Wheeler, A.W. and Woroniecki, S.R. (2001) Immunological adjuvants in allergy vaccines: Past, present and future. *Allergol Int*, **50**, 295-301.

Wild, C., Wallner, M., Hufnagl, K., Fuchs, H., Hoffmann-Sommergruber, K., Breiteneder, H., Scheiner, O., Ferreira, F. and Wiedermann, U. (2007) A recombinant allergen chimer as novel mucosal vaccine candidate for prevention of multi-sensitivities. *Allergy*, **62**, 33-41.

Ying Men, Gander, B., Merkle, H.P. and Corradin, G. (1996) Induction of sustained and elevated immune responses to weakly immunogenic synthetic malarial peptides by encapsulation in biodegradable polymer microspheres. *Vaccine*, **14**, 1442-1450.

CHAPTER 5

The coating of PLGA microparticles with protamine enhances their immunological performance through facilitated phagocytosis

The coating of PLGA microparticles with protamine enhances their immunological performance through facilitated phagocytosis

Julia M. Martínez Gómez[1], Anke Sichelstiel[1], Noémi Csaba[2], Stefan Fischer[2], Thomas M. Kündig[1], Bruno Gander[2], and Pål Johansen[1]

[1]Unit for Experimental Immunotherapy, Department of Dermatology, University Hospital Zurich, Zurich, Switzerland

[2]Drug Formulation and Delivery, Institute of Pharmaceutical Sciences, ETH Zurich, 8093 Zurich, Switzerland

J. Controlled release (accepted for publication)

ABSTRACT

Surface modifications of poly (lactide-co-glycolide) microparticles with different polycationic electrolytes have mainly been studied for conjugation to antigens and/or adjuvants. However, the *in vivo* immunological effects of using surface charged particles have not been address yet. In this study, microparticles were coated or not with protamine, a cationic and arginine-rich electrolyte that confers microparticles with a positively surface charge. We then evaluated the potential of protamine-coatings to assist the induction of immune responses in mice. Interestingly, enhanced antibodies and T-cell responses were observed in mice treated with the coated particles. *In vitro* studies suggested that the improved immunological performance was mediated by an increased uptake. Indeed, protamine-coated particles that carried a GFP-plasmid were even internalized into non-phagocytic cells and caused their transfection. These results open the way for further research into a novel technology that combines the use protamine for facilitated cell penetration and that of biodegradable microparticles for prolonged antigen or drug release.

INTRODUCTION

Polymeric biodegradable microparticles have been widely studied for a broad variety of pharmaceutical and biomedical applications (Johansen et al., 2007; Mundargi et al., 2007; Varde and Pack, 2004). Commonly used poly(lactide-co-glycolide) (PLGA) types are available in medical grade and are approved for use in humans, which makes them attractive for developing new drug and antigen delivery systems (Johansen et al., 2000; Langer, 1990; Putney and Burke, 1998). With a size range similar to that of microorganisms, polymeric particles can be easily taken up by antigen presenting cells (APCs) (Peyre et al., 2004b), a process that enables potent cellular as well as humoral immune responses (Ataman-Onal et al., 2006; Johansen et al., 1999; Men et al., 1995; Peter et al., 2001; Peyre et al., 2004a).

Physico-chemical properties such as the molecular weight and monomer composition of the polymer, and the size and surface charge of the microparticles determine the antigen release rate (Peyre et al., 2004a) and may also affect the type of immune response elicited by the delivery system (Espuelas S., 2005; Johansen et al., 2000). To add further flexibility and control of antigen and adjuvant delivery, the surface properties of PLGA microparticles have been modified (Fischer et al., 2007; Singh et al., 2004c; Singh et al., 2001). Surface modifications have been achieved with anionic electrolytes such as sodium dioctylsulfosuccinate (Singh et al., 2004a; Singh et al., 2004b) and cationic electrolytes such as chitosan, poly(ethylene imine), or protamine (Fischer et al., 2006; Fischer et al., 2007; Singh et al., 2004c; Singh et al., 2001). Protamine belongs to a group of low molecular weight (MW: 4000-4250 Da), arginine-rich, basic proteins, which condense DNA in the nucleus and are involved in spermatogenesis (Balhorn, 2007). Protamine is an FDA-approved compound, which has found applications in, e.g., stabilizing DNA (Elizabeth Collins, 2007), insulin complexation and formulation, and in reverting the anticoagulant effect of heparin (Brange and Langkjaer, 1992; Brange and Langkjaer, 1997; Byun Y, 2000). Protamine has been combined with PLGA microparticles for their use in mice (Dunne et al., 2003; Martinez Gomez et al., 2007) and used as a coating agent to bind nucleic acid on the surface of controlled-release particles (Csaba, 2007).

The aim of this study was to evaluate the properties of protamine-coated PLGA microparticles with regard to particle uptake, antigen presentation, and the induction of T-cell and antibody responses in mice.

MATERIALS AND METHODS

Materials

Purified phospholipase A_2 (PLA2) from bee venom and chicken egg albumin (OVA; grade V) were purchased from Sigma-Aldrich (Buchs, Switzerland), and soy bean lecithin (Epikuron 200) from Degussa (Hamburg, Germany). Phosphorothioate-modified CpG oligodeoxynucleotide 1668pt (5'-TCC-ATG-ACG-TTC-CCT-GAC-GTT-3') was synthesized by Microsynth (Balgach, Switzerland). The 35 kDa poly(lactide-co-glycolide) (PLGA 50:50) with uncapped end-groups (Resomer RG503H) was from Boehringer-Ingelheim (Ingelheim, Germany). Protamine sulphate from salmon, poly(vinyl alcohol) (PVA; Mowiol 4-88) and dichloromethane (DCM) were from Fluka (Buchs, Switzerland). The GFP plasmid (pmaxFP-Green-C) was provided by Amaxa (Cologne, Germany).

Mice

BALB/c, CBA/J and C57BL/6 mice were purchased from Harlan (Horst, the Netherlands) and used at 6-10 weeks of age. DO11.10 mice were generously provided by Martin F. Bachmann (Cytos Biotechnology, Switzerland) and are transgenic for the T-cell receptor recognizing the $OVA_{323-339}$ epitope on a $2H^d$ MHC-class II context in a BALB/c background (Murphy et al., 1990). OT-II mice were generously provided by Tobias Suter (University of Zurich, Switzerland). Also these mice are transgenic for the T-cell receptor recognizing the $OVA_{323-339}$ epitope but on a C57BL/6 ($2H^b$ MHC II) background (Barnden et al., 1998). All mice experiments were approved and performed according to guidelines formulated by the Veterinary authorities of the canton of Zurich, and the mice were kept in a specific pathogen free (SPF) environment.

Microparticle preparation

PLGA microparticles were made by microextrusion-based w/o/w-solvent extraction using a static multilamination type micromixer (Institut für Mikrotechnik Mainz, Mainz, Germany), as previously described (Freitas et al., 2003), with slight modifications. Three groups of formulations with different loads were prepared, with and without a protamine coating on the surface (Table 1). In the first group of microparticles, PLA2 was encapsulated for later evaluation of humoral responses. Briefly, 2 mg of PLA2 were dissolved in 100 µl water and emulsified on ice (ultrasonication with a probe for 10 seconds; 40% amplitude, UP200H, Hielscher Ultrasonics, Teltow, Germany) into 0.6 g of

PLGA dissolved in DCM (5%, w/w). This w/o-emulsion was extruded through the micromixer along with a 0.5% (w/w) PVA aqueous solution as extraction phase; the resulting suspension of microparticles was collected using the same aqueous solution of 0.5% (w/w) PVA for further solvent extraction and evaporation. To obtain positively charged microparticle coating, 0.5% (w/w) protamine solution was used as first extraction phase and water as final collection fluid. The particles were gently stirred using a magnetic rod and kept under laminar air-flow for 30 min for further solvent removal and hardening. Finally, the microparticles were collected on a mixed cellulose ester membrane filter with a pore size of 0.8 μm (Whatman, Dassel, Germany) and dried at 20 mbar and at room temperature for 24 h.

The second group of microparticles, with and without protamine coating, contained pGFP. They were prepared for analyzing particle uptake by cells and particle-mediated cell transfection. Briefly, pGFP (0.7 mg) was dissolved in Tris-EDTA (TE) buffer (0.2 ml) and emulsified in PLGA (0.2 g) dissolved in DCM (5%, w/w). The subsequent steps for microparticle formation were as described above.

The third group of microparticles, with and without protamine coating, was loaded with OVA for subsequently analyses of T-cell responses. Briefly, OVA (50 mg) was dissolved in PBS (0.8 ml), emulsified in PLGA (1 g) dissolved in DCM (5%, w/w), and further processed as described above.

Particle size and zeta potential measurements

Microparticle size distributions were determined by laser light diffraction (Mastersizer X, Malvern Instruments Malvern, United Kingdom) with a suspension of approx. 1 mg of dry microparticles in distilled water. A volume-weighted size distribution with 10%, 50% and 90% undersize diameters (D_{10}, D_{50} and D_{90}) was determined. The surface charge of the microparticles was determined by zeta potential measurement (Zetasizer 3000 HAS, Malvern Instruments). Dry microparticles (0.2 mg) were suspended in 1 mM KCl (2 ml; pH = 7.6), and the mean zeta potential value was calculated from two samples per batch and triplicate analysis per sample.

Internalization of pGFP-loaded PLGA microparticles (MP) by HEK cells

The role of the protamine in the particle uptake by cells was studied by measuring the transfection of Human Embryonic Kidney (HEK) cells upon incubation with microparticles containing the plasmid pGFP, taking use of the fact that positive transfection would

necessarily imply uptake of the plasmid containing particle by the cell; preliminary studies with FITC or coumarin-loaded particles were also done, but these did not allow to clearly distinguish between internalized particles and particles attached to the cell surface. Cells were seeded in 24-well plates pre-coated with poly-D-lysine at a density of $3x10^5$ per well. One day after seeding, 50, 100 or 200 μg of pGFP-MP or pGFP-MP/protamine were added to the cells in duplicates; after incubation for 4 hours and various subsequent washes, the cells were incubated with fresh complete medium. The transfection of cells was monitored for two further weeks using a Zeiss Axiovert fluorescence microscope (Carl Zeiss, Jena, Germany). The average number of transfected (green) cells in 10 randomly selected bright light fields was counted.

In vitro analysis of T–cell stimulation

Murine dendritic cells were isolated from BALB/c bone-marrow and grown in 10 cm Petri dishes at a density of 1×10^6 cell/ml in 10 ml RPMI-1640 complete media containing 20 ng/ml of granulocyte macrophage colony stimulating factor (GM-CSF) and 10 ng/ml of IL-4 (Immunotools, Friesoythe, Germany). After 2 and 4 days, the culture medium was replaced by fresh medium containing GM-CSF and IL-4. After 6 days, immature DCs were collected and seeded at 1×10^5 cells per well in a 96-well plate and then pulsed for 3 h at 37°C and 5% CO_2 with escalating amounts of OVA-containing microparticles, empty microparticles (1 or 100 μg) or aqueous OVA (10 or 100 μg/ml). After three washing steps, the cells were co-cultured with splenocytes ($1x10^5$ cells/well) from DO11.10 mice. After 20 h, aliquots of the supernatant were taken for IL-2 determination. Lymphocyte proliferation was measured after 48 h by [^{3}H] thymidine (1 μCi/well) incorporation during the last 16 h of culture using a 1450-MicroBeta TriLux liquid scintillation counter (Wallac, Finnland).

Immunization of mice

To evaluate the potential of the microparticles to stimulate T-cell responses *in vivo*, 6-10 week old female OT-II mice were used. The mice were injected with 1 mg of OVA-MP or similar particles coated with protamine (OVA-MP/protamine); for injection, the microparticles were suspended in 1% aqueous lecithin solution. Blood was withdrawn at different time points (7, 14 and 21 days), and the lymphocytes analyzed by flow cytometry for IFN-γ producing cells.

For the study of antibody responses, PLA2-containing microparticle formulations were tested in 6-10 weeks old female CBA/J mice. The formulations PLA2-MP or PLA2-MP/protamine were suspended in 1% aqueous lecithin solution prior to subcutaneous injection of a particle dose equivalent to 1 µg of entrapped PLA2; 1.2 - 1.3 mg of microparticles were injected in a volume of 150 µl. The mice were boosted with the same preparation and dose after 28 days. Serum was prepared from blood taken on days 28, 55 and 84 and frozen at -20°C until analyzed by ELISA. Additional two groups of mice were injected with the same two formulations PLA2-MP or PLA2-MP/protamine after addition of 3.1 nmole CpG.

Flow cytometry

Intracellular IFN-γ was assessed in blood of the OT-II mice injected with 1 mg of OVA-MP or OVA-MP/protamine. After lysis of the erythrocytes with hypotonic Red Blood Cell Lysis Buffer (Sigma-Aldrich), splenocytes were stimulated *in vitro* with soluble OVA (10 µg/ml) and brefeldin A (10 µg/ml) (Sigma-Aldrich) for 3 h at 37 °C and 5% CO_2. For FACS staining, the cells were washed several times, incubated with anti-CD16/CD32 for Fc-receptor blocking (5 min), and stained with anti-CD44-FITC and anti-CD4-APC antibodies for 30 min. Cells were then fixed with 1% paraformaldehyde for 10 min and subsequently stained with anti-IFN-γ-PE in PBS/0.5% saponine for 30 min. All stainings were done in PBS/2% FCS at 4°C, unless specified otherwise, and all antibodies were purchased from BD Pharmingen (San Diego, CA, USA). Samples were measured on a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA), and data was analyzed using FlowJo software (Ashland, OR, USA)

Antibody determination by enzyme-linked immunosorbent assay (ELISA)

For detection of PLA2 antibodies, microtitre 96-well plates (Nunc Maxisorb, Wiesbaden, Germany) were coated with 5 µg/ml PLA2 in carbonate buffer at 4 °C overnight. The plates were blocked with 2.5% skinned dry milk in phosphate-buffered saline containing 0.05% Tween 20 (Serva, Heidelberg, Germany) (PBSTM) for 1 h. Then, serial dilutions of individual mice sera in PBSTM were incubated in the plates for 2 h. Next, the plates were incubated with 1 µg/ml biotinylated goat anti-mouse IgG1 or IgG2a (BD Bioscience Pharmingen) in PBSTM for 2 h. Subsequently, plates were incubated with a 1:1000 dilution of streptavidin-conjugated horse-radish peroxidase (BD Pharmingen) for 1 h and developed with the enzyme substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-

sulphonic acid) diammonium salt (Sigma-Aldrich) in 1 M sodium dihydrogenphosphate. The endpoint optical density was measured at 405 nm after 20 min incubation using a microplate reader Model 550 (BioRad, Hercules, CA, USA). All steps were intercepted by washings with PBST.

RESULTS

Microparticle characterization

Three different groups of microparticles, all with and without protamine coating, were prepared (Table 1). The formulations with encapsulated pGFP, named pGFP-MP or pGFP-MP/protamine, had a very similar size distribution. Fifty percent of the particles were smaller than 3.2 μm or 3.5 μm, respectively. For both OVA- and PLA2-containing formulations, the size of the protamine-coated particles was larger than that of the non-coated particles. This was most evident for the OVA-containing microparticles, where 50% of the OVA-MP/protamine were smaller than 7.2 μm as compared to 3.2 μm for the non-coated particles (Table 1). Independent of the encapsulated antigen, the zeta potential of the protamine-coated microparticles was, as expected, positive (+11 to +65 mV), which is due to the polycationic charge of protamine; on the contrary, the non-coated particles had negative zeta potential (ranging from -11.8 to -2.4 mV).

Table 1. Microparticle formulations. Characteristics and application of the different groups of formulations used in the current the study.

Test formulation	**Zeta potential (mV)**	**Size Distribution (μm) ($D_{10}/D_{50}/D_{90}$)**	**Study application**
pGFP-MP	-11.8 ±0.8	1.6 / 3.2 / 5.2	Particle uptake and transfection
pGFP-MP/protamine	64.8 ± 4.3	1.9 / 3.5 / 5.3	
OVA-MP	-11.6 ± 0.1	1.4 / 3.2 / 6.4	Antigen presentation and T cell responses
OVA-MP/protamine	19.3 ± 0.6	2.3 / 7.2 / 20.9	
PLA2-MP	-2.4 ± 4.2	1.2 / 4.4 / 24.9	B cell responses
PLA2-MP/protamine	10.5 ± 6.6	3.0 / 6.8 / 15.5	

In vitro internalization of pGFP-loaded microparticles

To examine the effect of the protamine coating on the internalization of the microparticles, HEK cells were incubated for 4 h with different amounts of pGFP-containing particles, with or without protamine coating. Transfection of the cells was assessed as a measure for particle internalization by fluorescence microscopy. As illustrated in Figure 1A, only protamine-coated particles were able to transfect HEK cells.

The transfection was dose-dependent, as the efficacy increased with the amount of particles used in the culture (Fig. 1B).

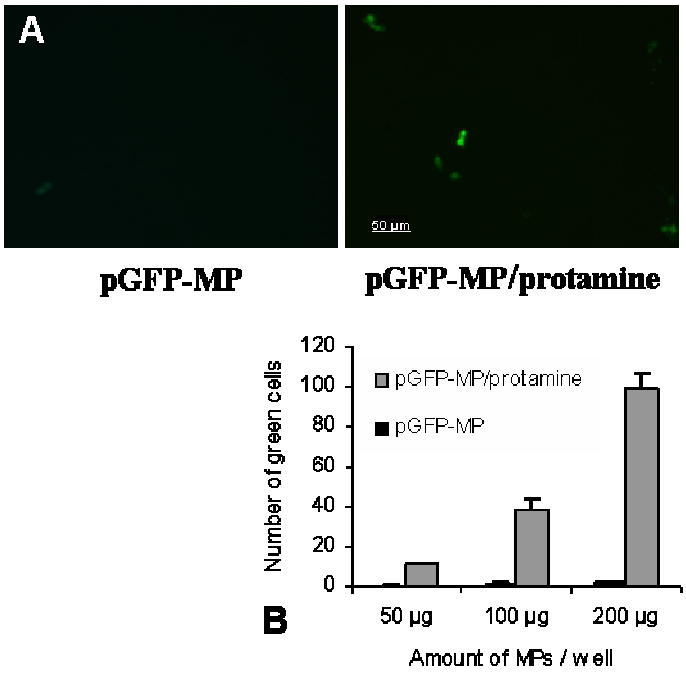

Figure 1. Internalization of microparticles containing pGFP by HEK cells. (A) Fluorescence microscopy pictures of cells incubated with 100 µg of protamine-coated or uncoated microparticles. Scale marker is 50 µm. (B) Quantification of positive cells as assessed by counting the green cells in 10 bright randomly selected light fields per well, after 72 h of incubation with the microparticles. The data is representative of three similar experiments.

In vitro proliferation of specific T cells

To assess the role of the protamine coating in the presentation of encapsulated antigen, OVA-containing microparticles, with and without protamine coating, were incubated at escalating amounts with bone-marrow derived DCs from BALB/c mice. The pulsed DCs were then co-cultured with splenocytes derived from transgenic DO11.10 mice. After 20 hours, the secretion of IL-2 in the supernatant was measured by ELISA and after 48 hours proliferation was determined by measuring the [^{3}H] thymidine incorporation. Figure 2A reveals that the protamine coating enhanced the IL-2 secretion in a dose-dependent manner and with a 10-fold higher sensitivity than the uncoated particles. Correspondingly, the protamine coated particles also showed a 10-fold increased capacity to stimulate the proliferation of antigen-specific T cells (Fig. 2B). The highest concentration of protamine-coated particles stimulated the secretion of IFN-γ (data not shown). This effect of protamine was also observed when compared to stimulation of splenocytes using DCs pulsed with soluble OVA. Cells stimulated with 100 µg/ml or 10 µg/ml OVA solution

produced very low or even undetectable levels of IL-2 (Fig. 2A), and moderate and concentration-dependent T-cell proliferation (Fig. 2B). Unloaded microparticles, with or without the protamine coating, did neither stimulate IL-2 production nor lymphocyte proliferation (data not shown).

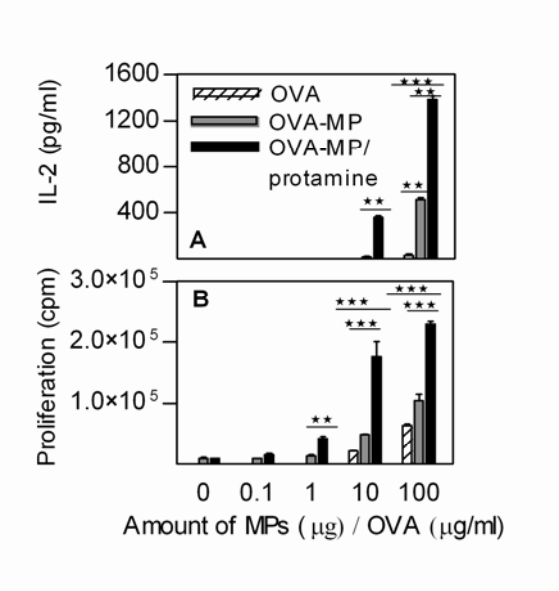

Figure 2. *In vitro* stimulation of OVA specific T cells. Bone marrow derived DCs from BALB/c mice were pulsed for 3 h with different amounts of OVA-loaded microparticles and then incubated with splenocytes from DO11.10 mice in tripiclates. After 20 h, IL-2 was measured in the supernatants (A), and after 48 h, cell proliferation was determined by [^{3}H] thymidine incorporation (B). Data was analyzed by Two Way ANOVA with Bonferroni post test (*** $p<0.0001$; ** $p<0.01$).

Stimulation of T-cell responses in mice

The capacity of microparticles to enhance the induction of T-cell responses *in vivo* was evaluated in transgenic OT-II mice. Animals were injected with 1 mg of either uncoated or protamine-coated microparticles containing OVA protein, OVA-MP or OVA-MP/protamine, and T-cell responses were assessed by measuring the activated CD4 T cells producing IFN-γ after 7, 14 and 21 days. Mice injected with OVA-MP/protamine induced higher frequencies of IFN-γ producing CD4 T cells at all time points as compared to mice that received OVA-MP (Fig. 3).

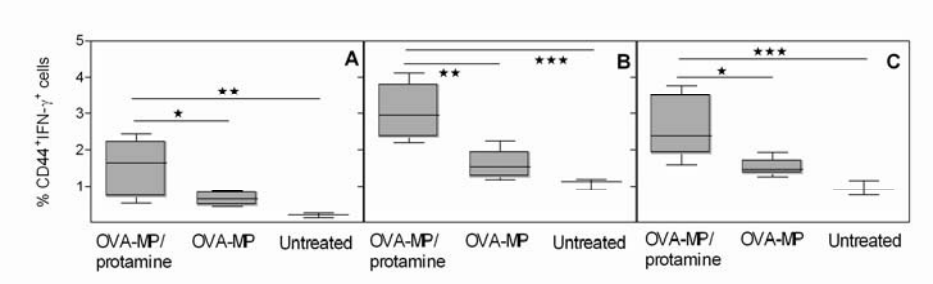

Figure 3. *In vivo* induction of T cell responses. OT-II mice were immunized with microparticles containing OVA (n=5), and the percentage of IFN-γ producing CD4 T cells in peripheral blood was measured by flow cytometry after 7 (A), 14 (B) and 21 (C) days. Untreated mice were used as negative controls. Data was analyzed by One Way ANOVA with Tukey's Multiple Comparison Test (*** $p<0.0001$; ** $p<0.01$; * $p<0.05$).

As early as day 7 (Fig. 3A) the protamine-coated microparticles induced significant levels of IFN-γ producing cells, in contrast to the uncoated microparticles that needed an additional week to raise a moderate response. At day 14, less than 2% of the cells from the mice immunized with the uncoated particles were IFN-γ producing CD4 T cells as compared to 3% achieved with the protamine-coated particles (Fig. 3B). Maximal response was attained with both formulations at day 14 after the injection (Fig. 3B). Untreated mice were used to determine the background levels of IFN-γ in the OT-II mice.

Stimulation of B-cell responses in mice

To evaluate the effect of the protamine coating on the stimulation of B-cell responses, CBA/J mice were immunized with microparticles containing the major bee venom allergen PLA2. The antibody responses were determined at 28, 55 and 84 days after the first injection. After the first injection, the protamine-coated particles induced a weak PLA2-specific IgG1 response, while the uncoated particles failed to do so, as measured 28 days after the priming (Fig. 4A). When a second dose was administered at day 28, the IgG1 response was strongly boosted by the protamine-coated particles, but not by the uncoated particles. The IgG1 levels remained high for at least 84 days. Two injections were required to induce a detectable IgG2a response; and again, the response, although weak, was stronger in mice immunized with the protamine-coated particles (Fig. 4C).

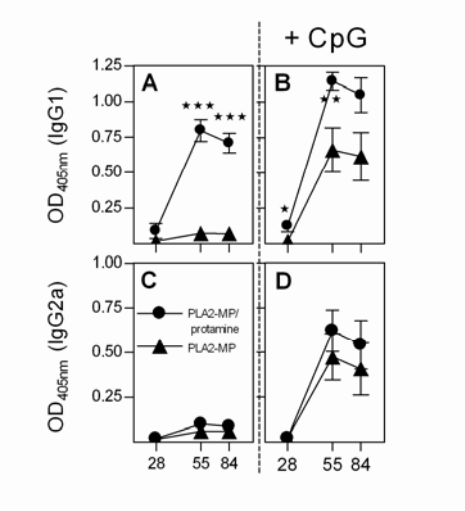

Figure 4. *In vivo* stimulation of humoral responses. CBA/J mice were immunized at day 0 and boosted at day 28 with PLA2-containing microparticles, with or without protamine coating, (A,C) and admixed with CpG (B,D) (n=4). Blood was collected at days 28, 55 and 84, and analyzed by ELISA for anti- PLA2 IgG1 (A,B) and IgG2a (C, D) antibodies. The absorbance at 1/800 serum dilution was chosen for comparing the groups at each time point. Data was analyzed by One Way ANOVA with Tukey's Multiple Comparison Test (*** $p<0.0001$; ** $p<0.01$; * $p<0.05$).

For assessing whether the observed benefit of the protamine coating persisted in the presence of an additional adjuvant, the two PLA2-loaded microparticle formulations were admixed with immune stimulatory CpG prior to injection. The previously observed Th2-biased response was shifted in the presence of CpG towards Th1, with both PLA2-MP and PLA2-MP/protamine (Fig. 4D). The IgG1 levels increased for both formulations, but the protamine-coated particles again stimulated stronger responses than the uncoated particles (Fig. 4B).

DISCUSSION

Microparticles of PLGA have been widely studied for their use as drug and antigen delivery systems (Johansen et al., 2000; Mundargi et al., 2007; Storni et al., 2005). These particulate systems are able to deliver drugs or antigens such as DNA, proteins, and peptides over prolonged periods of time, and to induce strong immune responses against encapsulated antigens (Espuelas S., 2005), which makes them attractive candidates for vaccine development.

Stability issues with encapsulated biologicals have led to an alternative method for associating antigens and DNA with PLGA microparticles, namely by adsorbing the bioactive materials onto the surface of anionic or cationic particles (Singh et al., 2004b; Singh et al., 2004c). Yet, not many studies have addressed the question if and to what extent the modified particle surface itself influences the induction of immune responses *in vivo*. In the current study, we investigated the immunological effects of coating PLGA microparticles with the cationic electrolyte protamine for stimulation of humoral and cellular immune responses to various antigens.

In mice immunized with microparticles containing the bee venom major allergen PLA2, we found that protamine-coated particles were more immunogenic than uncoated particles and enhanced the induction of long-lasting IgG1 antibody responses. In mice, the IgG1 subclass is associated with Th2 responses and is important to fight extracellular pathogens. In contrast, IgG2a is associated with Th1 and cytotoxic T-cell responses that are essential for inflammatory processes and in controlling intracellular pathogens. Although the protamine-coated particles also stimulated stronger IgG2a antibody responses than the uncoated microparticles, the overall IgG2a/Th1 response was weak, but could be strongly improved by admixing immunostimulatory CpG. In the presence of CpG, protamine-coated microparticles promoted again a stronger immune response than the uncoated particles. This capacity of polarizing the immune responses towards Th1 or Th2, as a function of the co-formulation with immune-response modifiers, may be exploited for different vaccination strategies, depending on the desired outcome.

The improved humoral immune responses after immunization with the protamine-coated microparticles prompted us to study whether protamine also affected the stimulation of T cells. Indeed, enhanced T-cell responses to ovalbumin were observed in mice after using OVA-containing particles coated with protamine. Although, differences were not as

striking as for the humoral responses, there was a significant increase in the percentage of IFN-γ producing CD4 T cells at all time points examined when compared to the uncoated particles. The enhanced immunogenicity of the protamine-coated particles was mediated through their preferential interaction with professional antigen-presenting cells. Bone-marrow derived DCs pulsed with protamine-coated microparticles stimulated antigen-specific T cells *in vitro* more efficient than did DCs pulsed with uncoated microparticles. One potential reason for the difference between protamine-coated and protamine-free particles with respect to their in interaction with APCs could have been differences in particle size distribution of the formulations. In this study, the size distribution of the uncoated OVA-containing microparticles was ideal for uptake, since 90% of the particles were smaller than 6.4 μm, and only particles smaller than 5-10 μm have been shown to be efficiently taken up by antigen presenting cells (Men et al., 1999; Peyre et al., 2004b). However, as the protamine-coated microparticles had a broader size distribution than the uncoated ones, with only 50% of the particles being smaller than 7.2 μm, the size cannot explain the increased *in vitro* and *in vivo* immune responses of the protamine-coated particles.

Besides particle size, particle surface charge may also influence the interaction with APCs. Positively charged particles are more likely to bind to the negatively charged cell surfaces, and thereby facilitate uptake and stimulation of immune responses. Indeed, cationic microparticles have been reported to enhance phagocytosis by DCs and macrophages in comparison with unmodified particles (Thiele et al., 2003; Thiele et al., 2001; Wischke et al., 2006). The mechanism suggested for the enhanced uptake in those studies is the electrostatic attraction between the positively charged microparticles and the negatively charged cell surface mediating binding and subsequent internalization. In addition to its positive charge at physiological pH, protamine specifically contains arginine-rich sequences, which share structural similarity with certain viral proteins such as the Tat from the Human Immunodeficiency Virus (HIV-1) or the VP22 from the Herpes Simplex Virus, both of which possess protein translocation activity (Futaki et al., 2001; Jarver and Langel, 2006; Schwarze et al., 2000). These sequences of basic amino acids mediate the penetration of viruses into cells in an energy-independent manner (Lundberg and Langel, 2003); thus, these so-called cell penetrating peptides or peptidic moieties appear to be particularly good candidates as transporters for drug delivery. While the cell penetrating property of Tat peptides have been extensively studied (Brooks et al., 2005;

Fittipaldi and Giacca, 2005; Torchilin, 2008), the potential of protamine to carry cargos across the cell membrane is less recognized. However, in 2005, two independent groups reported that protamine can act as an efficient membrane-translocating peptide both *in vitro* (Reynolds et al., 2005) and *in vivo* (Park et al., 2005; Reynolds et al., 2005). Park et al. showed that a protein toxin conjugated to protamine was able to translocate into cells *in vitro*, resulting in the inhibition of tumor growth *in vivo*. It was also reported that Tat-carrying (Lewin et al., 2000) and protamine-carrying (Reynolds et al., 2005) iron nanoparticles were readily internalized by cells. Moreover, Tat-liposomes of 200 nm showed potential for intracellular gene delivery *in vitro* and *in vivo* (Torchilin and Levchenko, 2003; Torchilin et al., 2003). These experiments suggest that the improved immunological performance of protamine-coated antigen-containing PLGA microparticles was mediated by the cell-penetrating properties of the arginine-rich protamine. To confirm this hypothesis we tested the uptake and transfection of non-phagocytic HEK cells using microparticles that contained a plasmid coding for GFP with or without protamine coating. Indeed, protamine-coated particles were capable to transfect the HEK cells, whereas uncoated particles did not possess this capacity. The same was observed when testing the particles on non-phagocytic melanoma cells (data not shown). As the size distribution of the tested formulations was almost identical (±0.3 μm), the observed differences must be ascribed to protamine and its ability to translocate the microparticles intracellularly.

Although, there is certain controversy in the literature concerning the translocation mechanisms of cell-penetrating peptides, recent data suggests more than one mechanism. Studies using Tat-nanoparticles or Tat-liposomes suggest that the translocation mechanism occurs via energy-dependent macropinocytosis (Gupta et al., 2005; Torchilin, 2008), whereas peptide-conjugated small molecules penetrate via electrostatic interactions and do not seem to depend on energy (Herce and Garcia, 2007). The exact mechanism of protamine-mediated penetration of microparticles into non-phagocytic cells remains to be clarified.

Taken together, we have shown the increased immunogenicity of antigens encapsulated in protamine-coated PLGA microparticles as compared to uncoated particles and proposed a possible mechanism of action. Additional research to characterize protamine-coated particles is required to develop further a new technology combining the advantages of microparticles for prolonged antigen release and protamine for facilitating cell penetration of the delivery system. The potential applications of such an intracellular delivery system

technology may span the areas of vaccine development, cancer immunotherapy, and gene/protein delivery.

Acknowledgements

The authors thank María J. Pena Rodríguez (University Hospital Zurich) for technical assistance, Martin Bachmann (Cytos Biotechnology) and Tobias Suter (Institute of Clinical Immunology, University of Zurich) for providing the transgenic mice, and Prof. Adriano Aguzzi (Institute of Neuropathology, University of Zurich) for helpful discussions.

Author contributions

This work is the result of a fruitful collaboration with the Institute of Drug Formulation and Delivery, from Pharmaceutical Sciences, ETH Zurich.

Together with Stefan Fischer and Noémi Csaba, I prepared the different microparticle formulations and partly characterized the microparticles *in vitro* at the ETH. I performed all *in vivo* and *in vitro* experiments and analyzed the data. After the paper got reviewed, Anke Sichestiel performed some more *in vitro* analysis of the microparticles.

Pål Johansen took part in the experimental design of the project and guided me through the writing of the manuscript. Bruno Gander and Thomas Kündig helped designing the experiments and corrected the manuscript.

REFERENCES

Ataman-Onal, Y., Munier, S., Ganee, A., Terrat, C., Durand, P.-Y., Battail, N., Martinon, F., Le Grand, R., Charles, M.-H., Delair, T. and Verrier, B. (2006) Surfactant-free anionic PLA nanoparticles coated with HIV-1 p24 protein induced enhanced cellular and humoral immune responses in various animal models. *Journal of Controlled Release*, **112**, 175-185.

Balhorn, R. (2007) The protamine family of sperm nuclear proteins. *Genome Biol*, **8**, 227.

Barnden, M.J., Allison, J., Heath, W.R. and Carbone, F.R. (1998) Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol*, **76**, 34-40.

Brange, J. and Langkjaer, L. (1992) Chemical stability of insulin. 3. Influence of excipients, formulation, and pH. *Acta Pharm Nord*, **4**, 149-158.

Brange, J. and Langkjaer, L. (1997) Insulin formulation and delivery. *Pharm Biotechnol*, **10**, 343-409.

Brooks, H., Lebleu, B. and Vives, E. (2005) Tat peptide-mediated cellular delivery: back to basics. *Adv Drug Delivery Rev*, **57**, 559.

Byun Y, C.L., Lee LM, Han IS, Singh VK, Yang VC . . (2000) Low molecular weight protamine: a potent but nontoxic antagonist to heparin/low molecular weight protamine. *ASAIO J.*, **46** 435-439.

Csaba, N.F., S; Gorodyska, G; Textor, M; Merkle, HP. (2007) Surface Modified Microparticles As Carriers For Nucleic Acid Vaccines And Immunopotentiators. *European Cells and Materials* **14**, 122.

Dunne, M., Bibby, D.C., Jones, J.C. and Cudmore, S. (2003) Encapsulation of protamine sulphate compacted DNA in polylactide and polylactide-co-glycolide microparticles. *Journal of Controlled Release*, **92**, 209.

Elizabeth Collins, J.C.B.J.L.W.M.G. (2007) Nuclear localisation and pDNA condensation in non-viral gene delivery. *The Journal of Gene Medicine*, **9**, 265-274.

Espuelas S., I.J.M., Gamazo C. (2005) Synthetic particulate antigen delivery systems for vaccination. *Inmunología*, **24**, 208-223.

Fischer, S., Foerg, C., Ellenberger, S., Merkle, H.P. and Gander, B. (2006) One-step preparation of polyelectrolyte-coated PLGA microparticles and their functionalization with model ligands. *Journal of Controlled Release*, **111**, 135.

Fischer, S., Uetz-von Allmen, E., Waeckerle-Men, Y., Groettrup, M., Merkle, H.P. and Gander, B. (2007) The preservation of phenotype and functionality of dendritic cells upon phagocytosis of polyelectrolyte-coated PLGA microparticles. *Biomaterials*, **28**, 994-1004.

Fittipaldi, A. and Giacca, M. (2005) Transcellular protein transduction using the Tat protein of HIV-1. *Adv Drug Deliv Rev*, **57**, 597-608.

Freitas, S., Walz, A., Merkle, H.P. and Gander, B. (2003) Solvent extraction employing a static micromixer: a simple, robust and versatile technology for the microencapsulation of proteins. *J Microencapsul*, **20**, 67-85.

Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K. and Sugiura, Y. (2001) Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J Biol Chem*, **276**, 5836-5840.

Gupta, B., Levchenko, T.S. and Torchilin, V.P. (2005) Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. *Adv Drug Delivery Rev*, **57**, 637-651.

Herce, H.D. and Garcia, A.E. (2007) Molecular dynamics simulations suggest a mechanism for translocation of the HIV-1 TAT peptide across lipid membranes. *Proc Natl Acad Sci U S A*, **104**, 20805-20810.

Jarver, P. and Langel, U. (2006) Cell-penetrating peptides--A brief introduction. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, **1758**, 260-263.

Johansen, P., Martinez Gomez, J.M. and Gander, B. (2007) Development of synthetic biodegradable microparticulate vaccines: a roller coaster story. *Expert Rev Vaccines*, **6**, 471-474.

Johansen, P., Men, Y., Merkle, H.P. and Gander, B. (2000) Revisiting PLA/PLGA microspheres: an analysis of their potential in parenteral vaccination. *Eur J Pharm Biopharm*, **50**, 129-146.

Johansen, P., Moon, L., Tamber, H., Merkle, H.P., Gander, B. and Sesardic, D. (1999) Immunogenicity of single-dose diphtheria vaccines based on PLA/PLGA microspheres in guinea pigs. *Vaccine*, **18**, 209-215.

Langer, R. (1990) New methods of drug delivery. *Science*, **249**, 1527-1533.

Lewin, M., Carlesso, N., Tung, C.H., Tang, X.W., Cory, D., Scadden, D.T. and Weissleder, R. (2000) Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells. *Nat Biotechnol*, **18**, 410-414.

Lundberg, P. and Langel, U. (2003) A brief introduction to cell-penetrating peptides. *J Mol Recognit*, **16**, 227-233.

Martinez Gomez, J.M., Fischer, S., Csaba, N., Kundig, T.M., Merkle, H.P., Gander, B. and Johansen, P. (2007) A Protective Allergy Vaccine Based on CpG- and Protamine-Containing PLGA Microparticles. *Pharm Res*, **24**, 1927-1935.

Men, Y., Audran, R., Thomasin, C., Eberl, G., Demotz, S., Merkle, H.P., Gander, B. and Corradin, G. (1999) MHC class I- and class II-restricted processing and presentation of microencapsulated antigens. *Vaccine*, **17**, 1047-1056.

Men, Y., Thomasin, C., Merkle, H.P., Gander, B. and Corradin, G. (1995) A single administration of tetanus toxoid in biodegradable microspheres elicits T cell and antibody responses similar or superior to those obtained with aluminum hydroxide. *Vaccine*, **13**, 683-689.

Mundargi, R.C., Babu, V.R., Rangaswamy, V., Patel, P. and Aminabhavi, T.M. (2007) Nano/micro technologies for delivering macromolecular therapeutics using poly(d,l-lactide-co-glycolide) and its derivatives. *J Control Release*.

Murphy, K.M., Heimberger, A.B. and Loh, D.Y. (1990) Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science*, **250**, 1720-1723.

Park, Y.J., Chang, L.-C., Liang, J.F., Moon, C., Chung, C.-P. and Yang, V.C. (2005) Nontoxic membrane translocation peptide from protamine, low molecular weight protamine (LMWP), for enhanced intracellular protein delivery: in vitro and in vivo study. *FASEB J.*, **19**, 1555-1557.

Peter, K., Men, Y., Pantaleo, G., Gander, B. and Corradin, G. (2001) Induction of a cytotoxic T-cell response to HIV-1 proteins with short synthetic peptides and human compatible adjuvants. *Vaccine*, **19**, 4121-4129.

Peyre, M., Audran, R., Estevez, F., Corradin, G., Gander, B., Sesardic, D. and Johansen, P. (2004a) Childhood and malaria vaccines combined in biodegradable microspheres produce immunity with synergistic interactions. *J Control Release*, **99**, 345-355.

Peyre, M., Fleck, R., Hockley, D., Gander, B. and Sesardic, D. (2004b) In vivo uptake of an experimental microencapsulated diphtheria vaccine following sub-cutaneous immunisation. *Vaccine*, **22**, 2430-2437.

Putney, S.D. and Burke, P.A. (1998) Improving protein therapeutics with sustained-release formulations. *Nat Biotechnol*, **16**, 153-157.

Reynolds, F., Weissleder, R. and Josephson, L. (2005) Protamine as an efficient membrane-translocating peptide. *Bioconjug Chem*, **16**, 1240-1245.

Schwarze, S.R., Hruska, K.A. and Dowdy, S.F. (2000) Protein transduction: unrestricted delivery into all cells? *Trends in Cell Biology*, **10**, 290.

Singh, M., Chesko, J., Kazzaz, J., Ugozzoli, M., Kan, E., Srivastava, I. and O'Hagan, D.T. (2004a) Adsorption of a novel recombinant glycoprotein from HIV (Env gp120dV2 SF162) to anionic PLG microparticles retains the structural integrity of the protein, whereas encapsulation in PLG microparticles does not. *Pharm Res*, **21**, 2148-2152.

Singh, M., Kazzaz, J., Chesko, J., Soenawan, E., Ugozzoli, M., Giuliani, M., Pizza, M., Rappouli, R. and O'Hagan, D.T. (2004b) Anionic microparticles are a potent delivery system for recombinant antigens from Neisseria meningitidis serotype B. *J Pharm Sci*, **93**, 273-282.

Singh, M., Kazzaz, J., Ugozzoli, M., Chesko, J. and O'Hagan, D.T. (2004c) Charged polylactide co-glycolide microparticles as antigen delivery systems. *Expert Opin Biol Ther*, **4**, 483-491.

Singh, M., Ott, G., Kazzaz, J., Ugozzoli, M., Briones, M., Donnelly, J. and O'Hagan, D.T. (2001) Cationic microparticles are an effective delivery system for immune stimulatory cpG DNA. *Pharm Res*, **18**, 1476-1479.

Storni, T., Kundig, T.M., Senti, G. and Johansen, P. (2005) Immunity in response to particulate antigen-delivery systems. *Adv Drug Deliv Rev*, **57**, 333-355.

Thiele, L., Merkle, H.P. and Walter, E. (2003) Phagocytosis and phagosomal fate of surface-modified microparticles in dendritic cells and macrophages. *Pharm Res*, **20**, 221-228.

Thiele, L., Rothen-Rutishauser, B., Jilek, S., Wunderli-Allenspach, H., Merkle, H.P. and Walter, E. (2001) Evaluation of particle uptake in human blood monocyte-derived cells in vitro. Does phagocytosis activity of dendritic cells measure up with macrophages? *J Control Release*, **76**, 59-71.

Torchilin, V.P. (2008) Tat peptide-mediated intracellular delivery of pharmaceutical nanocarriers. *Advanced Drug Delivery Reviews*, **60**, 548-558.

Torchilin, V.P. and Levchenko, T.S. (2003) TAT-liposomes: a novel intracellular drug carrier. *Curr Protein Pept Sci*, **4**, 133-140.

Torchilin, V.P., Levchenko, T.S., Rammohan, R., Volodina, N., Papahadjopoulos-Sternberg, B. and D'Souza, G.G. (2003) Cell transfection in vitro and in vivo with nontoxic TAT peptide-liposome-DNA complexes. *Proc Natl Acad Sci U S A*, **100**, 1972-1977.

Varde, N.K. and Pack, D.W. (2004) Microspheres for controlled release drug delivery. *Expert Opin Biol Ther*, **4**, 35-51.

Wischke, C., Borchert, H.-H., Zimmermann, J., Siebenbrodt, I. and Lorenzen, D.R. (2006) Stable cationic microparticles for enhanced model antigen delivery to dendritic cells. *Journal of Controlled Release*, **114**, 359-368.

ACKNOWLEDGMENTS

Foremost, I would like to thank Thomas Kündig for giving me the opportunity to do my PhD in his lab and for being always so supportive and generous with me. It has been a great chance to be in a lab where both pre-clinical and clinical research are in close contact. I think I could never find the right words to describe how grateful I am to Pål Johansen who has constantly supported and assisted me not only in the lab but also with the writing of the various manuscripts that have come out from this PhD. In Spanish you would say: "Tienes más paciencia que un santo". Mange takk.

I am also very thankful to Prof. Adriano Aguzzi for accepting me as an external PhD student and for his helpful input. I would also like to thank Bruno Gander who agreed first to start a collaboration between our two groups and second to be my co-referee. Bruno, you have always been ready to lend a hand and full of ideas to improve the project, thank you.

Thanks also to Stefan Fisher and Noémi Csaba, both from the institute of Pharmaceutical Science, for the great collaboration and for always being so kind and helpful.

My gratitude also goes to Gabriela Senti, without your organizational skills the group would be lost!

I would also like to thank the "F14 club" that started with Giulia, Natalie and later on Ossia, it has been great to have so much support from you not only in the lab but also in the office, where we had our long chats about the ups and downs of a PhD and in general about everything. A thanks goes also to our new PhD students, Anke and Antonia who are full of energy and always ready to help, I am sure you will do a great job.

I thank all the people working in the clinic of dermatology, specially the people in the F floor. Susi muchísimas gracias por ayudarme con las megaELISAs, sin ti todavía seguiría lavando platos!

Thanks a lot to all my dearest friends for being always there for me.

Muchísimas gracias a las chicas de los martes, Feli, Nadia, Cris y Sabine, qué voy a hacer sin vosotras cuando me vaya. Habéis sido un gran apoyo para mi, con vosotras he compartido desde lágrimas hasta risas, sobre todo esto último.

A mi familia gracias por vuestro gran apoyo incondicional, incluso a miles de kilómetros siempre me habéis apoyado en todas mis decisiones, gracias de todo corazón.

CURRICULUM VITAE

Name: Julia M. Martínez Gómez

E-mail: juliam@bluewin.ch / julia.martinez@usz.ch

Birth date: 9 January 1977

Nationality: Spanish

EDUCATION

2004-2008 PhD thesis at the University Zurich
Subject: Novel Treatments for Allergen-Specific Immunotherapy

2001-2002 Diploma Thesis at the University of Zurich, Division of Psychiatric Research
Subject: Antibody-mediated uptake of fibrillar Aβ by microglial cells *in vitro*

1995-2000 Master of Science, Biochemistry at the University of Salamanca (Spain)

1991-1995 Tomas Edison High School in Albacete (Spain)

COURSES ATTENDED

April 2007 "2nd ENII-Mugen Summer School in Advanced Immunology", Capo Caccia

July 2005 "Proteomics", ETH, Zurich

March 2004 "Introductory Course in Laboratory Animal Science" LTK Module 1E, Zürich

Nov. 2003 "Flow cytometry", BD Bioscience, Madrid

CONGRESSES ATTENDED

2007 Oral presentation at the SGAI, Basel, Switzerland.
Oral presentation at the EAACI, Göteborg, Sweden.
Poster at the EAACI, Göteborg, Sweden.
Poster at Day of Clinical Research.
Poster at the 2nd ENII-Mugen summer school in advance immunology.
Poster at World Immune Regulation Meeting, Davos, Switzerland.

2006 Oral presentation at the SGAI, Zurich, Switzerland.

Oral presentation at the European Academy of Allergy and Clinical immunology (EAACI), Vienna, Austria.

Poster at 4th EAACI GA2LEN Davos Meeting, Grainau, Germany.

Poster at 5th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Geneva, Switzerland.

2005 Poster at Swiss Society for Allergology and Immunology (SGAI), Bern, Switzerland.

PUBLICATIONS

Martinez Gomez, J.M., Fischer, S., Csaba, N., Kundig, T.M., Merkle, H.P., Gander, B. and Johansen, P. (2007) A Protective Allergy Vaccine Based on CpG- and Protamine-Containing PLGA Microparticles. *Pharm Res*, **24**, 1927-1935.

Martinez Gomez, J.M., Johansen, P, Horst, R., Steiner, M., Senti, G., Rhyner, C., Crameri, R., and Kundig, T.M. (2008) Targeting the MHC class II pathway of antigen presentation enhances immunogenicity and safety of allergen immunotherapy. *Allergy* (accepted for publication).

Martinez Gomez, J.M., Sichelstiel, A., Csaba, N., Fischer, S., Kundig, T.M., Gander, B. and Johansen, P. (2008) The coating of PLGA microparticles with protamine enhances their immunological performance through facilitated phagocytosis. Accepted for publication in *J Control Release*.

Martinez Gomez, J.M., Johansen, P, Erdmann, I., Crameri, R., and Kundig, T.M. (2008) Intralymphatic injections as a new prospective administration route for allergen immunotherapy. Submitted for publication.

Johansen, P., **Martinez Gomez, J.M**. and Gander, B. (2007) Development of synthetic biodegradable microparticulate vaccines: a roller coaster story. *Expert Rev Vaccines*, **6**, 471-474.

Johansen, P., Senti, G., **Martinez Gomez, J.M.**, Storni, T., von Beust, B.R., Wuthrich, B., Bot, A. and Kundig, T.M. (2005a) Toll-like receptor ligands as adjuvants in allergen-specific immunotherapy. *Clin Exp Allergy*, **35**, 1591-1598.

Johansen, P., Senti, G., **Martinez Gomez, J.M**., Wuthrich, B., Bot, A. and Kundig, T.M. (2005b) Heat denaturation, a simple method to improve the immunotherapeutic potential of allergens. *Eur J Immunol*, **35**, 3591-3598.

Mohajeri, M.H., Gaugler, M.N., **Martinez, J.**, Tracy, J., Li, H., Crameri, A., Kuehnle, K., Wollmer, M.A. and Nitsch, R.M. (2004) Assessment of the bioactivity of antibodies against beta-amyloid peptide in vitro and in vivo. *Neurodegener Dis*, **1**, 160-167.

Senti, G., Johansen, P., **Martinez Gomez, J**., Prinz Varicka, B.M. and Kundig, T.M. (2005) Efficacy and safety of allergen-specific immunotherapy in rhinitis, rhinoconjunctivitis, and bee/wasp venom allergies. *Int Rev Immunol*, **24**, 519-531.

Senti, G., **Martinez Gomez, J.M**., Rettig, L., Wuthrich, B., Kundig, T.M. and Johansen, P. (2006) Immunotherapeutic targeting of allergic disease. *Inflamm Allergy Drug Targets*, **5**, 243-252.

WORK EXPERIENCE

Jan. 03-Aug. 04	Glycart Biotechnology, Schlieren (CH)**.** Research associate
Jan. 01-April 01	Genexcel Irl. Ltd, West Cork, Ireland. Laboratory assistant
Nov. 00-Feb. 01	Biochemistry department, University College Cork, Cork, Ireland Demonstrator

REFEREES

Dr. Thomas Kündig
Unit for Experimental Immunotherapy
Gloriastr. 31
8091 Zurich (CH)
Tel: 004112553973 e-mail: Thomas.Kuendig@usz.ch

Dr. Pablo Umaña
CSO of Glycart Biotechnology
Wagistrasse 18
8952 Schlieren-Zurich (CH)
Tel/Fax: 004117556161 e-mail: Pablo.umana@glycart.com

Dr. Hasan Mohajeri/ Dr. Axel Wollmer
Division of Psychiatric Research
August-Forelstr. 1
8032 Zürich (CH)
Tel/Fax: 004116348888 e-mail: mohajeri@bli.unizh.ch wollmer@bli.unizh.ch